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TITLE OF THE INVENTION (500 characters max)			
METHODS AND COMPOSITIONS FOR TARGETED CLEAVAGE AND RECOMBINATION			
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[Page 1 of 2]

Respectfully submitted,

Date March 26, 2004

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April Reeves

**METHODS AND COMPOSITIONS FOR TARGETED CLEAVAGE
AND RECOMBINATION**

CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

STATEMENT OF RIGHTS TO INVENTIONS

MADE UNDER FEDERALLY SPONSORED RESEARCH

Not applicable.

TECHNICAL FIELD

The present disclosure is in the field of genome engineering and homologous recombination.

BACKGROUND

A major area of interest in genome biology, especially in light of the determination of the complete nucleotide sequences of a number of genomes, is the targeted alteration of genome sequences. To provide but one example, sickle cell anemia is caused by mutation of a single nucleotide pair in the human β -globin gene. Thus, the ability to convert the endogenous genomic copy of this mutant nucleotide pair to the wild-type sequence in a stable fashion and produce β -globin would provide a cure for sickle cell anemia.

Attempts have been made to alter genomic sequences by taking advantage of the natural phenomenon of homologous recombination. *See*, for example, Capecchi (1989)

Science **244**:1288-1292; U.S. Patent Nos. 6,528,313 and 6,528,314. If a polynucleotide has sufficient homology to the genomic region containing the sequence to be altered, it is possible for part or all of the sequence of the polynucleotide to replace the genomic sequence by homologous recombination. However, the frequency of homologous recombination under these circumstances is extremely low. Moreover, the frequency of insertion of the exogenous polynucleotide at genomic locations that lack sequence homology exceeds the frequency of homologous recombination by several orders of magnitude.

The introduction of a double-stranded break into genomic DNA, in the region of the genome bearing homology to an exogenous polynucleotide, has been shown to stimulate homologous recombination at this site by several thousand-fold. Rouet *et al.* (1994) *Mol. Cell. Biol.* **14**:8096-8106; Chouluka *et al.* (1995) *Mol. Cell. Biol.* **15**:1968-1973; Donoho *et al.* (1998) *Mol. Cell. Biol.* **18**:4070-4078. See also Johnson *et al.* (2001) *Biochem. Soc. Trans.* **29**:196-201; and Yanez *et al.* (1998) *Gene Therapy* **5**:149-159. In these methods, DNA cleavage in the desired genomic region was accomplished by inserting a recognition site for a meganuclease (*i.e.*, an endonuclease whose recognition sequence is so large that it does not occur, or occurs only rarely, in the genome of interest) into the desired genomic region.

However, meganuclease cleavage-stimulated homologous recombination relies on either the fortuitous presence of, or the directed insertion of, a suitable meganuclease recognition site in the vicinity of the genomic region to be altered. Since meganuclease recognition sites are rare (or nonexistent) in a typical mammalian genome, and insertion of a suitable meganuclease recognition site is plagued with the same difficulties as associated with other genomic alterations, these methods are not broadly applicable.

Thus, there remains a need for compositions and methods for targeted replacement of sequences in any genome.

SUMMARY

The present disclosure provides compositions and methods for targeted cleavage of cellular chromatin in a region of interest and/or targeted homologous recombination in cells. A region of interest in cellular chromatin can be, for example, a genomic sequence

or portion thereof. Compositions include fusion polypeptides comprising an engineered zinc finger binding domain (*e.g.*, a zinc finger binding domain having a novel specificity) and a cleavage domain, and fusion polypeptides comprising an engineered zinc finger binding domain and a cleavage half-domain. Cleavage domains and cleavage half domains can be obtained, for example, from various restriction endonucleases and/or homing endonucleases.

Cellular chromatin can be present in any type of cell including, but not limited to, prokaryotic and eukaryotic cells, fungal cells, plant cells, animal cells, mammalian cells, primate cells and human cells.

In one aspect, a method for cleavage of cellular chromatin in a region of interest (*e.g.*, a method for targeted cleavage of genomic sequences) is provided, the method comprising: (a) selecting a first sequence in the region of interest; (b) engineering a first zinc finger binding domain to bind to the first sequence; and (c) expressing a first fusion protein in the cell, the first fusion protein comprising the first engineered zinc finger binding domain and a cleavage domain; wherein the first fusion protein binds to the first sequence and the cellular chromatin is cleaved in the region of interest. The site of cleavage can be coincident with the sequence to which the fusion protein binds, or it can be adjacent (*e.g.*, separated from the near edge of the binding site by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more nucleotides). A fusion protein can be expressed in a cell, *e.g.*, by delivering the fusion protein to the cell or by delivering a polynucleotide encoding the fusion protein to a cell, wherein the polynucleotide is transcribed, and the transcript is translated, to generate the fusion protein. Methods for polynucleotide and polypeptide delivery to cells are presented elsewhere in this disclosure.

In certain embodiments, the cleavage domain may comprise two cleavage half-domains that are covalently linked in the same polypeptide. The two cleavage half-domains can be derived from the same endonuclease or from different endonucleases.

In additional embodiments, targeted cleavage of cellular chromatin in a region of interest is achieved by expressing two fusion proteins in a cell, each fusion protein comprising a zinc finger binding domain and a cleavage half-domain. One or both of the zinc finger binding domains of the fusion proteins can be engineered to bind to a target sequence in the vicinity of the cleavage site. If expression of the fusion proteins is by

polynucleotide delivery, each of the two fusion proteins can be encoded by a separate polynucleotide, or a single polynucleotide can encode both fusion proteins.

Accordingly, a method for cleaving cellular chromatin in a region of interest can comprise (a) selecting a first sequence in the region of interest; (b) engineering a first zinc finger binding domain to bind to the first sequence; (c) expressing a first fusion protein in the cell, the first fusion protein comprising the first zinc finger binding domain and a first cleavage half-domain; and (d) expressing a second fusion protein in the cell, the second fusion protein comprising a second zinc finger binding domain and a second cleavage half-domain, wherein the first fusion protein binds to the first sequence, and the second fusion protein binds to a second sequence located between 2 and 50 nucleotides from the first sequence, thereby positioning the cleavage half-domains such that the cellular chromatin is cleaved in the region of interest.

In certain embodiments, binding of the first and second fusion proteins positions the cleavage half-domains such that a functional cleavage domain is reconstituted.

In certain embodiments, the second zinc finger binding domain is engineered to bind to the second sequence. In further embodiments, the first and second cleavage half-domains are derived from the same endonuclease, which can be, for example, a restriction endonuclease (*e.g.*, a Type IIS restriction endonuclease such as *Fok I*) or a homing endonuclease.

In other embodiments, any of the methods described herein may comprise (a) selecting first and second sequences in a region of interest, wherein the first and second sequences are between 2 and 50 nucleotides apart; (b) engineering a first zinc finger binding domain to bind to the first sequence; (c) engineering a second zinc finger binding domain to bind to the second sequence; (d) expressing a first fusion protein in the cell, the first fusion protein comprising the first engineered zinc finger binding domain and a first cleavage half-domain; (e) expressing a second fusion protein in the cell, the second fusion protein comprising the second engineered zinc finger binding domain and a second cleavage half-domain; wherein the first fusion protein binds to the first sequence and the second fusion protein binds to the second sequence, thereby positioning the first and second cleavage half-domains such that the cellular chromatin is cleaved in the region of interest.

In certain embodiments, the first and second cleavage half-domains are derived from the same endonuclease, for example, a Type IIS restriction endonuclease, for example, *Fok I*. In additional embodiments, cellular chromatin is cleaved at one or more sites between the first and second sequences to which the fusion proteins bind.

5 In further embodiments, a method for cleavage of cellular chromatin in a region of interest comprises (a) selecting the region of interest; (b) engineering a first zinc finger binding domain to bind to a first sequence in the region of interest; (c) providing a second zinc finger binding domain which binds to a second sequence in the region of interest, wherein the second sequence is located between 2 and 50 nucleotides from the first
10 sequence; (d) expressing a first fusion protein in the cell, the first fusion protein comprising the first zinc finger binding domain and a first cleavage half-domain; and (e) expressing a second fusion protein in the cell, the second fusion protein comprising the second zinc finger binding domain and a second cleavage half domain; wherein the first fusion protein binds to the first sequence, and the second fusion protein binds to the
15 second sequence, thereby positioning the cleavage half-domains such that the cellular chromatin is cleaved in the region of interest.

In any of the methods described herein, the first and second cleavage half-domains may be derived from the same endonuclease or from different endonucleases. In additional embodiments, the second zinc finger binding domain is engineered to bind to
20 the second sequence.

If one or more polynucleotides encoding the fusion proteins are introduced into the cell, an exemplary method for targeted cleavage of cellular chromatin in a region of interest comprises (a) selecting the region of interest; (b) engineering a first zinc finger binding domain to bind to a first sequence in the region of interest; (c) providing a second
25 zinc finger binding domain which binds to a second sequence in the region of interest, wherein the second sequence is located between 2 and 50 nucleotides from the first sequence; and (d) contacting a cell with (i) a first polynucleotide encoding a first fusion protein, the fusion protein comprising the first zinc finger binding domain and a first cleavage half-domain, and (ii) a second polynucleotide encoding a second fusion protein,
30 the fusion protein comprising the second zinc finger binding domain and a second cleavage half domain; wherein the first and second fusion proteins are expressed, the first

fusion protein binds to the first sequence and the second fusion protein binds to the second sequence, thereby positioning the cleavage half-domains such that the cellular chromatin is cleaved in the region of interest. In a variation of this method, a cell is contacted with a single polynucleotide which encodes both fusion proteins.

5 For any of the aforementioned methods, the cellular chromatin can be in a chromosome, episome or organellar genome. In addition, in any of the methods described herein, at least one zinc finger binding domain is engineered, for example by design or selection methods.

Similarly, for any of the aforementioned methods, the cleavage half domain can
10 be derived from, for example, a homing endonuclease or a restriction endonuclease, for example, a Type IIS restriction endonuclease. An exemplary Type IIS restriction endonuclease is *Fok I*.

Methods for targeted recombination (*e.g.*, for replacement of a sequence in a chromosome or a region of interest in cellular chromatin) are also provided. For
15 example, a mutant genomic sequence can be replaced by a wild-type sequence, *e.g.*, for treatment of genetic disease or inherited disorders. In addition, a wild-type genomic sequence can be replaced by a mutant sequence, *e.g.*, to prevent function of an oncogene product or a product of a gene involved in an inappropriate inflammatory response.

Accordingly, in one aspect, a method for replacement of a region of interest in
20 cellular chromatin (*e.g.*, a genomic sequence) with a first nucleotide sequence is provided, the method comprising: (a) engineering a zinc finger binding domain to bind to a second sequence in the region of interest; (b) expressing a fusion protein in a cell, the fusion protein comprising the zinc finger binding domain and a cleavage domain; and (c) contacting the cell with a polynucleotide comprising the first nucleotide sequence;
25 wherein the fusion protein binds to the second sequence such that the cellular chromatin is cleaved in the region of interest and the region of interest is replaced with the first nucleotide sequence. Generally, cellular chromatin is cleaved in the region of interest at or adjacent to the second sequence. In further embodiments, the cleavage domain comprises two cleavage half-domains, which can be derived from the same or from
30 different nucleases.

In addition, a method for replacement of a region of interest in cellular chromatin (*e.g.*, a genomic sequence) with a first nucleotide sequence is provided, the method comprising: (a) engineering a first zinc finger binding domain to bind to a second sequence in the region of interest; (b) providing a second zinc finger binding domain to bind to a third sequence in the region of interest; (c) expressing a first fusion protein in a cell, the first fusion protein comprising the first zinc finger binding domain and a first cleavage half-domain; (d) expressing a second fusion protein in the cell, the second fusion protein comprising the second zinc finger binding domain and a second cleavage half-domain; and (e) contacting the cell with a polynucleotide comprising the first nucleotide sequence; wherein the first fusion protein binds to the second sequence and the second fusion protein binds to the third sequence, thereby positioning the cleavage half-domains such that the cellular chromatin is cleaved in the region of interest and the region of interest is replaced with the first nucleotide sequence. Generally, cellular chromatin is cleaved in the region of interest at a site between the second and third sequences.

Additional methods for replacement of a region of interest in cellular chromatin (*e.g.*, a genomic sequence) with a first nucleotide sequence comprise: (a) selecting a second sequence, wherein the second sequence is in the region of interest and has a length of at least 9 nucleotides; (b) engineering a first zinc finger binding domain to bind to the second sequence; (c) selecting a third sequence, wherein the third sequence has a length of at least 9 nucleotides and is located between 2 and 50 nucleotides from the second sequence; (d) providing a second zinc finger binding domain to bind to the third sequence; (e) expressing a first fusion protein in a cell, the first fusion protein comprising the first zinc finger binding domain and a first cleavage half-domain; (f) expressing a second fusion protein in the cell, the second fusion protein comprising the second zinc finger binding domain and a second cleavage half-domain; and (g) contacting the cell with a polynucleotide comprising the first nucleotide sequence; wherein the first fusion protein binds to the second sequence and the second fusion protein binds to the third sequence, thereby positioning the cleavage half-domains such that the cellular chromatin is cleaved in the region of interest and the region of interest is replaced with the first

nucleotide sequence. Generally, cellular chromatin is cleaved in the region of interest at a site between the second and third sequences.

In another aspect, methods for targeted recombination are provided in which, a first nucleotide sequence, located in a region of interest in cellular chromatin, is replaced with a second nucleotide sequence. The methods comprise (a) engineering a first zinc finger binding domain to bind to a third sequence in the region of interest; (b) providing a second zinc finger binding domain to bind to a fourth sequence; (c) expressing a first fusion protein in a cell, the fusion protein comprising the first zinc finger binding domain and a first cleavage half-domain; (d) expressing a second fusion protein in the cell, the second fusion protein comprising the second zinc finger binding domain and a second cleavage half-domain; and (e) contacting a cell with a polynucleotide comprising the second nucleotide sequence; wherein the first fusion protein bind to the third sequence and the second fusion protein binds to the fourth sequence, thereby positioning the cleavage half-domains such that the cellular chromatin is cleaved in the region of interest and the first nucleotide sequence is replaced with the second nucleotide sequence.

In the aforementioned methods for targeted recombination, the binding sites for the fusion proteins (*i.e.*, the third and fourth sequences) can comprise any number of nucleotides. Preferably, they are at least nine nucleotides in length, but they can also be larger (*e.g.*, 10, 11, 12, 13, 14, 15, 16, 17, 18 and up to 100 nucleotides, including any integral value between 9 and 100 nucleotides); moreover the third and fourth sequences need not be the same length. The distance between the binding sites (*i.e.*, the length of nucleotide sequence between the third and fourth sequences) can be any integral number of nucleotide pairs between 2 and 50, as measured from the near end of one binding site to the near end of the other binding site.

In the aforementioned methods for targeted recombination, cellular chromatin can be cleaved at a site located between the binding sites of the two fusion proteins. Moreover, expression of the fusion proteins in the cell can be accomplished either by introduction of the proteins into the cell or by introduction of one or more polynucleotides into the cell, which are transcribed, and the transcript(s) translated, to produce the fusion proteins. For example, two polynucleotides, each comprising sequences encoding one of the two fusion proteins, can be introduced into a cell.

Alternatively, a single polynucleotide comprising sequences encoding both fusion proteins can be introduced into the cell.

Thus, in one embodiment, a method for replacement of a region of interest in cellular chromatin (*e.g.*, a genomic sequence) with a first nucleotide sequence comprises:

- 5 (a) engineering a first zinc finger binding domain to bind to a second sequence in the region of interest; (b) providing a second zinc finger binding domain to bind to a third sequence; and (c) contacting a cell with:
 - (i) a first polynucleotide comprising the first nucleotide sequence;
 - (ii) a second polynucleotide encoding a first fusion protein, the first fusion protein
 - 10 comprising the first zinc finger binding domain and a first cleavage half-domain; and
 - (iii) a third polynucleotide encoding a second fusion protein, the second fusion protein comprising the second zinc finger binding domain and a second cleavage half-domain;

wherein the first and second fusion proteins are expressed, the first fusion protein

- 15 binds to the second sequence and the second fusion protein binds to the third sequence, thereby positioning the cleavage half-domains such that the cellular chromatin is cleaved in the region of interest; and the region of interest is replaced with the first nucleotide sequence.

- In any of the methods described herein, the first nucleotide sequence (the “donor sequence”) may be homologous, but not identical, to the genomic sequence that is replaced, thereby stimulating homologous recombination to insert a non-identical sequence in the region of interest. Thus, in certain embodiments, the donor sequence exhibits between about 80 to 99% (or any integer therebetween) sequence identity to the genomic sequence that is replaced. In other embodiments, the homology between the
- 20 donor and genomic sequence is higher than 99%, for example if only 1 nucleotide differs as between donor and genomic sequences of over 100 contiguous base pairs. Although the non-identical portion of the donor sequence can be anywhere within the donor sequence, it is typically located internally so as to be flanked by regions of homology. In other embodiments, the donor sequence is non-homologous to the first sequence, and is
 - 25 inserted into the genome by non-homologous recombination mechanisms.
 - 30

In methods for targeted recombination and/or replacement of a sequence of interest in cellular chromatin, the first and second cleavage half-domains can be derived from the same endonuclease or from different endonucleases. Endonucleases include, but are not limited to, homing endonucleases and restriction endonucleases. Exemplary restriction endonucleases are Type IIS restriction endonucleases; an exemplary Type IIS restriction endonuclease is *Fok I*.

The region of interest can be in a chromosome, episome or organellar genome. The region of interest can comprise a mutation, which can be replaced by a wild type sequence (or by a different mutant sequence), or the region of interest can contain a wild-type sequence that is replaced by a mutant sequence. Mutations include, but are not limited to, point mutations (transitions, transversions), insertions of one or more nucleotide pairs, deletions of one or more nucleotide pairs, rearrangements, inversions and translocation. Mutations can change the coding sequence, introduce premature stop codon(s) and/or modify the frequency of a repetitive sequence motif (e.g., trinucleotide repeat) in a gene. For applications in which targeted recombination is used to replace a mutant sequence, cellular chromatin is generally cleaved at a site located within 100 nucleotides on either side of the mutation.

In any of the methods described herein, the second zinc finger binding domain can be engineered, for example designed and/or selected.

Further, the donor polynucleotide can be DNA or RNA, can be linear or circular, and can be single-stranded or double-stranded. It can be delivered to the cell as naked nucleic acid, as a complex with one or more delivery agents (e.g., liposomes, poloxamers) or contained in a viral delivery vehicle, such as, for example, an adenovirus or an adeno-associated Virus (AAV). Donor sequences can range in length from 10 to 1,000 nucleotides (or any integral value of nucleotides therebetween) or longer.

Similarly, polynucleotides encoding fusions between a zinc finger binding domain and a cleavage domain or half-domain can be DNA or RNA, can be linear or circular, and can be single-stranded or double-stranded. They can be delivered to the cell as naked nucleic acid, as a complex with one or more delivery agents (e.g., liposomes, poloxamers) or contained in a viral delivery vehicle, such as, for example, an adenovirus

or an adeno-associated Virus (AAV). A polynucleotide can encode one or more fusion proteins.

In the methods for targeted recombination, as with the methods for targeted cleavage, a cleavage domain or half-domain can be derived from any nuclease, *e.g.*, a homing endonuclease or a restriction endonuclease, in particular, a Type IIS restriction endonuclease. Cleavage half-domains can be derived from the same or from different endonucleases. An exemplary source, from which a cleavage half-domain can be derived, is the Type IIS restriction endonuclease *Fok I*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a portion of the human hSMC1 gene encoding the amino-terminal portion of the protein (SEQ ID NO:**) and the encoded amino acid sequence (SEQ ID NO: **). Target sequences for the hSMC1-specific ZFPs are underlined.

Figure 2 shows a schematic diagram of a plasmid encoding a ZFP-FokI fusion for targeted cleavage of the hSMC1 gene.

Figure 3 A-D show a schematic diagram of the hSMC1 gene. **Figure 3A** shows a schematic of a portion of the human X chromosome which includes the hSMC1 gene. **Figure 3B** shows a schematic of a portion of the hSMC1 gene including the upstream region (left of +1), the first exon (between +1 and the right end of the arrow labeled "SMC1 coding sequence") and a portion of the first intron. Locations of sequences homologous to the initial amplification primers and to the chromosome-specific primer (see Table 3) are also provided. **Figure 3C** shows the sequence of the human X chromosome in the region of the SMC1 initiation codon (SEQ ID NO: **), the encoded amino acid sequence (SEQ ID NO: **), and the target sites for the SMC1-specific zinc finger proteins. **Figure 3D** shows the sequence of the corresponding region of the donor molecule, with differences between donor and chromosomal sequences underlined. Sequences contained in the donor-specific amplification primer (Table 3) are indicated by double underlining.

Figure 4 shows a schematic diagram of the hSMC1 donor construct.

Figure 5 shows PCR analysis of DNA from transfected HEK293 cells. From left, the lanes show results from cells transfected with a plasmid encoding GFP (control plasmid), cells transfected with two plasmids, each of which encodes one of the two hSMC1-specific ZFP-FokI fusion proteins (ZFPs only), cells transfected with two concentrations of the hSMC1 donor plasmid (donor only), and cells transfected with the two ZFP-encoding plasmids and the donor plasmid (ZFPs + donor). See Example 1 for details.

Figure 6 shows the nucleotide sequence of an amplification product derived from a mutated hSMC1 gene (SEQ ID NO:**) generated by targeted homologous recombination. Sequences derived from the vector into which the amplification product was cloned are single-underlined, chromosomal sequences not present in the donor molecule are indicated by dashed underlining (nucleotides 32-97), sequences common to the donor and the chromosome are not underlined (nucleotides 98-394 and 402-417), and sequences unique to the donor are double-underlined (nucleotides 395-401). Lower-case letters represent sequences that differ between the chromosome and the donor.

Figure 7 shows the nucleotide sequence of a portion of the human IL2R γ gene comprising the 3' end of the second intron and the 5' end of third exon (SEQ ID NO:**) and the amino acid sequence encoded by the displayed portion of the third exon (SEQ ID NO:**). Target sequences for the second pair of IL2R γ -specific ZFPs are underlined. See Example 2 for details.

Figure 8 shows a schematic diagram of a plasmid encoding a ZFP-FokI fusion for targeted cleavage of IL2R γ gene.

Figure 9 A-D show a schematic diagram of the IL2R γ gene. Figure 9A shows a schematic of a portion of the human X chromosome which includes the IL2R γ gene.

Figure 9B shows a schematic of a portion of the IL2R γ gene including a portion of the second intron, the third exon and a portion of the third intron. Locations of sequences homologous to the initial amplification primers and to the chromosome-specific primer (see Table 5) are also provided. Figure 9C shows the sequence of the human X chromosome in the region of the third exon of the IL2R γ gene (SEQ ID NO: **), the encoded amino acid sequence (SEQ ID NO: **), and the target sites for the first pair of IL2R γ -specific zinc finger proteins. Figure 9D shows the sequence of the corresponding

region of the donor molecule, with differences between donor and chromosomal sequences underlined. Sequences contained in the donor-specific amplification primer (Table 5) are indicated by double overlining.

Figure 10 shows a schematic diagram of the IL2R γ donor construct.

Figure 11 shows PCR analysis of DNA from transfected K652 cells. From left, the lanes show results from cells transfected with two plasmids, each of which encodes one of a pair of IL2R γ -specific ZFP-FokI fusion proteins (ZFPs only, lane 1), cells transfected with two concentrations of the IL2R γ donor plasmid (donor only, lanes 2 and 3), and cells transfected with the two ZFP-encoding plasmids and the donor plasmid (ZFPs + donor, lanes 4-7). Each of the two pairs of IL2R γ -specific ZFP-FokI fusions were used (identified as "pair 1" and "pair 2") and use of both pairs resulted in production of the diagnostic amplification product (labeled "expected chimeric product" in the Figure). See Example 2 for details.

Figure 12 shows the nucleotide sequence of an amplification product derived from a mutated IL2R γ gene (SEQ ID NO:**) generated by targeted homologous recombination. Sequences derived from the vector into which the amplification product was cloned are single-underlined, chromosomal sequences not present in the donor molecule are indicated by dashed underlining (nucleotides 460-552), sequences common to the donor and the chromosome are not underlined (nucleotides 32-42 and 59-459), and a stretch of sequence containing nucleotides which distinguish donor sequences from chromosomal sequences is double-underlined (nucleotides 44-58). Lower-case letters represent nucleotides whose sequence differs between the chromosome and the donor.

Figure 13 shows the nucleotide sequence of a portion of the human beta-globin gene encoding segments of the core promoter, the first two exons and the first intron (SEQ ID NO: **). A missense mutation changing an A at position 5212541 on Chromosome 11 (BLAT, UCSC Genome Bioinformatics site) to a T (in boldface and underlined) results in sickle cell anemia. A first zinc finger/FokI fusion protein was designed such that the primary contacts were with the underlined 12-nucleotide sequence AAGGTGAACGTG (SEQ ID NO: **), and a second zinc finger/FokI fusion protein was designed such that the primary contacts were with the complement of the underlined 12-nucleotide sequence CCGTTACTGCCC (SEQ ID NO: **).

Figure 14 is a schematic diagram of a plasmid encoding ZFP-FokI fusion for targeted cleavage of the human beta globin gene.

Figure 15 is a schematic diagram of the cloned human beta globin gene showing the upstream region, first and second exons, first intron and primer binding sites.

5 **Figure 16** is a schematic diagram of the beta globin donor construct, pCR4-TOPO-HBBdonor.

10 **Figure 17** shows PCR analysis of DNA from cells transfected with two pairs of β -globin-specific ZFP nucleases and a beta globin donor plasmid. The panel on the left is a loading control in which the initial amp 1 and initial amp 2 primers (Table 7) were used for amplification. In the experiment shown in the right panel, the "chromosome-specific and "donor-specific" primers (Table 7) were used for amplification. The leftmost lane in each panel contains molecular weight markers and the next lane shows amplification products obtained from mock-transfected cells. Remaining lanes, from left to right, show amplification product from cells transfected with: a GFP-encoding plasmid, 100ng of each ZFP/FokI-encoding plasmid, 200ng of each ZFP/FokI-encoding plasmid, 200 ng donor plasmid, 600 ng donor plasmid, 200 ng donor plasmid + 100 ng of each ZFP/FokI-encoding plasmid, and 600 ng donor plasmid + 200 ng of each ZFP/FokI-encoding plasmid.

20 **Figure 18** shows the nucleotide sequence of an amplification product derived from a mutated beta-globin gene (SEQ ID NO:**) generated by targeted homologous recombination. Chromosomal sequences not present in the donor molecule are indicated by dashed underlining (nucleotides 1-72), sequences common to the donor and the chromosome are not underlined (nucleotides 73-376), and a stretch of sequence containing nucleotides which distinguish donor sequences from chromosomal sequences is double-underlined (nucleotides 377-408). Lower-case letters represent nucleotides whose sequence differs between the chromosome and the donor.

Figure 19 shows the nucleotide sequence of a portion of the fifth exon of the Interleukin-2 receptor gamma chain (IL-2R γ) gene. Also shown are the target sequences for the 5-8 and 5-10 ZFP/FokI fusion proteins. See Example 5 for details.

30 **Figure 20** shows the amino acid sequence of the 5-8 ZFP/FokI fusion targeted to exon 5 of the human IL-2R γ gene (SEQ ID NO:**). Amino acid residues 1-17 contain a

nuclear localization sequence (NLS, underlined); residues 18-130 contain the ZFP portion, with the recognition regions of the component zinc fingers shown in boldface; the ZFP-*FokI* linker (ZC linker, underlined) extends from residues 131 to 140 and the *FokI* cleavage half-domain begins at residue 141 and extends to the end of the protein at residue 336. The residue that was altered to generate the Q486E mutation is shown underlined and in boldface.

Figure 21 shows the amino acid sequence of the 5-10 ZFP/*FokI* fusion targeted to exon 5 of the human IL-2R γ gene (SEQ ID NO: **). Amino acid residues 1-17 contain a nuclear localization sequence (NLS, underlined); residues 18-133 contain the ZFP portion, with the recognition regions of the component zinc fingers shown in boldface; the ZFP-*FokI* linker (ZC linker, underlined) extends from residues 134 to 143 and the *FokI* cleavage half-domain begins at residue 144 and extends to the end of the protein at residue 339. The residue that was altered to generate the E490K mutation is shown underlined and in boldface.

Figure 22 shows the nucleotide sequence of the enhanced Green Fluorescent Protein gene (SEQ ID NO: **) derived from the *Aequorea victoria* GFP gene (Tsien (1998) *Ann. Rev. Biochem.* 67:509-544). The ATG initiation codon, as well as the region which was mutagenized, are underlined.

Figure 23 shows the nucleotide sequence of a mutant defective eGFP gene (SEQ ID NO: **). Binding sites for ZFP-nucleases are underlined and the region between the binding sites corresponds to the region that was modified.

Figure 24 shows the structures of plasmids encoding Zinc Finger Nucleases targeted to the eGFP gene.

Figure 25 shows an autoradiogram of a 10% acrylamide gel used to analyze targeted DNA cleavage of a mutant eGFP gene by zinc finger endonucleases. See Example 8 for details.

Figure 26 shows the structure of plasmid pcDNA4/TO/GFPmut (see Example 9).

Figure 27 shows levels of eGFPmut mRNA, normalized to GAPDH mRNA, in various cell lines obtained from transfection of human HEK293 cells. Light bars show levels in untreated cells; dark bars show levels in cell that had been treated with 2 ng/ml doxycycline. See Example 9 for details.

Figure 28 shows the structure of plasmid pCR(R)4-TOPO-GFPdonor5. See Example 10 for details.

Figure 29 shows the nucleotide sequence of the eGFP insert in pCR(R)4-TOPO-GFPdonor5 (SEQ ID NO: **). The insert contains sequences encoding a portion of a non-modified enhanced Green Fluorescent Protein, lacking an initiation codon. See Example 10 for details.

Figure 30 shows a FACS trace of T18 cells transfected with plasmids encoding two ZFP nucleases and a plasmid encoding a donor sequence, that were arrested in the G2 phase of the cell cycle 24 hours post-transfection with 100 ng/ml nocodazole for 48 hours. The medium was replaced and the cells were allowed to recover for an additional 48 hours, and gene correction was measured by FACS analysis. See Example 11 for details.

Figure 31 shows a FACS trace of T18 cells transfected with plasmids encoding two ZFP nucleases and a plasmid encoding a donor sequence, that were arrested in the G2 phase of the cell cycle 24 hours post-transfection with 0.2 uM vinblastine for 48 hours. The medium was replaced and the cells were allowed to recover for an additional 48 hours, and gene correction was measured by FACS analysis. See Example 11 for details.

Figure 32 shows the nucleotide sequence of a 1,527 nucleotide eGFP insert in pCR(R)4-TOPO (SEQ ID NO: **). The sequence encodes a non-modified enhanced Green Fluorescent Protein lacking an initiation codon. See Example 13 for details.

Figure 33 shows a schematic diagram of an assay used to measure the frequency of editing of the endogenous human IL-2R γ gene. See Example 14 for details.

Figure 34 shows autoradiograms of acrylamide gels used in an assay to measure the frequency of editing of an endogenous cellular gene by targeted cleavage and homologous recombination. The lane labeled "GFP" shows assay results from a control in which cells were transfected with an eGFP-encoding vector; the lane labeled "ZFPs only" shows results from another control experiment in which cells were transfected with the two ZFP/nuclease-encoding plasmids (50 ng of each) but not with a donor sequence. Lanes labeled "donor only" show results from a control experiment in which cells were transfected with 1 ug of donor plasmid but not with the ZFP/nuclease-encoding plasmids.

In the experimental lanes, 50Z refers to cells transfected with 50 ng of each ZFP/nuclease expression plasmid, 100Z refers to cells transfected with 100 ng of each ZFP/nuclease expression plasmid, 0.5D refers to cells transfected with 0.5 μ g of the donor plasmid, and 1D refers to cells transfected with 1.0 μ g of the donor plasmid. "+" refers to cells that were exposed to 0.2 μ M vinblastine; "-" refer to cells that were not exposed to vinblastine. "wt" refers to the fragment obtained after *Bsr*BI digestion of amplification products obtained from chromosomes containing the wild-type chromosomal IL-2R γ gene; "rflp" refers to one of the two fragments obtained after *Bsr*BI digestion of amplification products obtained from chromosomes containing sequences from the donor plasmid which had integrated by homologous recombination.

DETAILED DESCRIPTION

Disclosed herein are compositions and methods useful for targeted cleavage of cellular chromatin and for targeted homologous recombination between an exogenous polynucleotide and a genomic sequence. Genomic sequences include those present in chromosomes, episomes, organellar genomes (e.g., mitochondria, chloroplasts), artificial chromosomes and any other type of nucleic acid present in a cell such as, for example, amplified sequences, double minute chromosomes and the genomes of endogenous or infecting bacteria and viruses. Chromosomes can be normal or mutant; mutant chromosomes can comprise, for example, insertions, deletions, translocations, rearrangements, and/or point mutations.

Compositions useful for targeted cleavage and recombination include fusion proteins comprising a cleavage domain (or a cleavage half-domain) and a zinc finger binding domain, polynucleotides encoding these proteins and combinations of polypeptides and polypeptide-encoding polynucleotides. A zinc finger binding domain can comprise one or more zinc fingers (e.g., 2, 3, 4, 5, 6, 7, 8, 9 or more zinc fingers), and can be engineered to bind to any genomic sequence. Thus, by identifying a target genomic region of interest at which cleavage or recombination is desired, one can, according to the methods disclosed herein, construct one or more fusion proteins comprising a cleavage domain (or cleavage half-domain) and a zinc finger domain engineered to recognize a target sequence in said genomic region. The presence of such a

fusion protein (or proteins) in a cell will result in binding of the fusion protein(s) to its (their) binding site(s) and cleavage within or near said genomic region. Moreover, if an exogenous polynucleotide homologous to the genomic region is also present in such a cell, targeted recombination occurs at a high rate between the genomic region and the exogenous polynucleotide.

General

Practice of the methods, as well as preparation and use of the compositions disclosed herein employ, unless otherwise indicated, conventional techniques in molecular biology, biochemistry, chromatin structure and analysis, computational chemistry, cell culture, recombinant DNA and related fields as are within the skill of the art. These techniques are fully explained in the literature. *See*, for example, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, Second edition, Cold Spring Harbor Laboratory Press, 1989 and Third edition, 2001; Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, 1987 and periodic updates; the series METHODS IN ENZYMOLOGY, Academic Press, San Diego; Wolffe, CHROMATIN STRUCTURE AND FUNCTION, Third edition, Academic Press, San Diego, 1998; METHODS IN ENZYMOLOGY, Vol. 304, "Chromatin" (P.M. Wassarman and A. P. Wolffe, eds.), Academic Press, San Diego, 1999; and METHODS IN MOLECULAR BIOLOGY, Vol. 119, "Chromatin Protocols" (P.B. Becker, ed.) Humana Press, Totowa, 1999.

Definitions

The terms "nucleic acid," "polynucleotide," and "oligonucleotide" are used interchangeably and refer to a deoxyribonucleotide or ribonucleotide polymer, in linear or circular conformation, and in either single- or double-stranded form. For the purposes of the present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogues of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties (*e.g.*, phosphorothioate backbones). In general, an analogue of a particular nucleotide has the same base-pairing specificity; *i.e.*, an analogue of A will base-pair with T.

The terms "polypeptide," "peptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. The term also applies to amino acid polymers in which one or more amino acids are chemical analogues or modified derivatives of a corresponding naturally-occurring amino acids.

- 5 "Binding" refers to a sequence-specific, non-covalent interaction between macromolecules (e.g., between a protein and a nucleic acid). Not all components of a binding interaction need be sequence-specific (e.g., contacts with phosphate residues in a DNA backbone), as long as the interaction as a whole is sequence-specific. Such interactions are generally characterized by a dissociation constant (K_d) of 10^{-6} M⁻¹ or
10 lower. "Affinity" refers to the strength of binding: increased binding affinity being correlated with a lower K_d .

- A "binding protein" is a protein that is able to bind non-covalently to another molecule. A binding protein can bind to, for example, a DNA molecule (a DNA-binding protein), an RNA molecule (an RNA-binding protein) and/or a protein molecule (a protein-
15 binding protein). In the case of a protein-binding protein, it can bind to itself (to form homodimers, homotrimers, etc.) and/or it can bind to one or more molecules of a different protein or proteins. A binding protein can have more than one type of binding activity. For example, zinc finger proteins have DNA-binding, RNA-binding and protein-binding activity.

- A "zinc finger DNA binding protein" (or binding domain) is a protein, or a domain
20 within a larger protein, that binds DNA in a sequence-specific manner through one or more zinc fingers, which are regions of amino acid sequence within the binding domain whose structure is stabilized through coordination of a zinc ion. The term zinc finger DNA binding protein is often abbreviated as zinc finger protein or ZFP.

- Zinc finger binding domains can be "engineered" to bind to a predetermined
25 nucleotide sequence. Non-limiting examples of methods for engineering zinc finger proteins are design and selection. A designed zinc finger protein is a protein not occurring in nature whose design/composition results principally from rational criteria. Rational criteria for design include application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP
30 designs and binding data. See, for example, US Patents 6,140,081; 6,453,242; and 6,534,261; see also WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and

WO 03/016496. Additional exemplary methods for identifying binding sites and engineering zinc finger binding domains are disclosed in co-owned US Patent Application entitled "Selection of target sequences for zinc finger proteins" (Reference No. S37-PR, Attorney Docket No. 019496-006900US), filed even date herewith.

5 A "selected" zinc finger protein is a protein not found in nature whose production results primarily from an empirical process such as phage display, interaction trap or hybrid selection. See *e.g.*, US 5,789,538; US 5,925,523; US 6,007,988; US 6,013,453; US 6,200,759; WO 95/19431; WO 96/06166; WO 98/53057; WO 98/54311; WO 00/27878; WO 01/60970 WO 01/88197 and WO 02/099084.

10 The term "sequence" refers to a nucleotide sequence of any length, which can be DNA or RNA; can be linear, circular or branched and can be either single-stranded or double stranded. The term "donor sequence" refers to a nucleotide sequence that is inserted into a genome. A donor sequence can be of any length, for example between 2 and 1,000 nucleotides in length (or any integer value therebetween), preferably between
15 about 100 and 1,000 nucleotides in length (or any integer therebetween), more preferably between about 200 and 500 nucleotides in length.

A "homologous, non-identical sequence" refers to a first sequence which shares a degree of sequence identity with a second sequence, but whose sequence is not identical to that of the second sequence. For example, a polynucleotide comprising the wild-type
20 sequence of a mutant gene is homologous and non-identical to the sequence of the mutant gene. In certain embodiments, the degree of homology between the two sequences is sufficient to allow homologous recombination therebetween, utilizing normal cellular mechanisms of homologous recombination. Two homologous non-identical sequences can be any length and their degree of non-homology can be as small as a single
25 nucleotide (*e.g.*, for correction of a genomic point mutation by targeted homologous recombination). Two polynucleotides comprising the homologous non-identical sequences need not be the same length. For example, an exogenous polynucleotide of between 20 and 1,000 nucleotides or nucleotide pairs can be used to correct a genomic point mutation.

30 Techniques for determining nucleic acid and amino acid sequence identity are known in the art. Typically, such techniques include determining the nucleotide

sequence of the mRNA for a gene and/or determining the amino acid sequence encoded thereby, and comparing these sequences to a second nucleotide or amino acid sequence. Genomic sequences can also be determined and compared in this fashion. In general, identity refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more sequences (polynucleotide or amino acid) can be compared by determining their percent identity. The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M.O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, WI) in the "BestFit" utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, WI). A preferred method of establishing percent identity in the context of the present disclosure is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects sequence identity. Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH

SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank
CDS translations + Swiss protein + Spupdate + PIR. Details of these programs can be
found at the following internet address: <http://www.ncbi.nlm.gov/cgi-bin/BLAST>. With
respect to sequences described herein, the range of desired degrees of sequence identity is
5 approximately 80% to 100% and any integer value therebetween. Typically the percent
identities between sequences are at least 70-75%, preferably 80-82%, more preferably
85-90%, even more preferably 92%, still more preferably 95%, and most preferably 98%
sequence identity.

Alternatively, the degree of sequence similarity between polynucleotides can be
10 determined by hybridization of polynucleotides under conditions that allow formation of
stable duplexes between homologous regions, followed by digestion with single-
stranded-specific nuclease(s), and size determination of the digested fragments. Two
nucleic acid, or two polypeptide sequences are substantially homologous to each other
when the sequences exhibit at least about 70%-75%, preferably 80%-82%, more
15 preferably 85%-90%, even more preferably 92%, still more preferably 95%, and most
preferably 98% sequence identity over a defined length of the molecules, as determined
using the methods above. As used herein, substantially homologous also refers to
sequences showing complete identity to a specified DNA or polypeptide sequence. DNA
sequences that are substantially homologous can be identified in a Southern hybridization
20 experiment under, for example, stringent conditions, as defined for that particular system.
Defining appropriate hybridization conditions is within the skill of the art. See, e.g.,
Sambrook et al., *supra*; Nucleic Acid Hybridization: A Practical Approach, editors B.D.
Hames and S.J. Higgins, (1985) Oxford; Washington, DC; IRL Press).

Selective hybridization of two nucleic acid fragments can be determined as
25 follows. The degree of sequence identity between two nucleic acid molecules affects the
efficiency and strength of hybridization events between such molecules. A partially
identical nucleic acid sequence will at least partially inhibit the hybridization of a
completely identical sequence to a target molecule. Inhibition of hybridization of the
completely identical sequence can be assessed using hybridization assays that are well
30 known in the art (e.g., Southern (DNA) blot, Northern (RNA) blot, solution
hybridization, or the like, see Sambrook, et al., *Molecular Cloning: A Laboratory*

Manual, Second Edition, (1989) Cold Spring Harbor, N.Y.). Such assays can be conducted using varying degrees of selectivity, for example, using conditions varying from low to high stringency. If conditions of low stringency are employed, the absence of non-specific binding can be assessed using a secondary probe that lacks even a partial degree of sequence identity (for example, a probe having less than about 30% sequence identity with the target molecule), such that, in the absence of non-specific binding events, the secondary probe will not hybridize to the target.

When utilizing a hybridization-based detection system, a nucleic acid probe is chosen that is complementary to a reference nucleic acid sequence, and then by selection of appropriate conditions the probe and the reference sequence selectively hybridize, or bind, to each other to form a duplex molecule. A nucleic acid molecule that is capable of hybridizing selectively to a reference sequence under moderately stringent hybridization conditions typically hybridizes under conditions that allow detection of a target nucleic acid sequence of at least about 10-14 nucleotides in length having at least approximately 70% sequence identity with the sequence of the selected nucleic acid probe. Stringent hybridization conditions typically allow detection of target nucleic acid sequences of at least about 10-14 nucleotides in length having a sequence identity of greater than about 90-95% with the sequence of the selected nucleic acid probe. Hybridization conditions useful for probe/reference sequence hybridization, where the probe and reference sequence have a specific degree of sequence identity, can be determined as is known in the art (see, for example, Nucleic Acid Hybridization: A Practical Approach, editors B.D. Hames and S.J. Higgins, (1985) Oxford; Washington, DC; IRL Press).

Conditions for hybridization are well-known to those of skill in the art. Hybridization stringency refers to the degree to which hybridization conditions disfavor the formation of hybrids containing mismatched nucleotides, with higher stringency correlated with a lower tolerance for mismatched hybrids. Factors that affect the stringency of hybridization are well-known to those of skill in the art and include, but are not limited to, temperature, pH, ionic strength, and concentration of organic solvents such as, for example, formamide and dimethylsulfoxide. As is known to those of skill in the art, hybridization stringency is increased by higher temperatures, lower ionic strength and lower solvent concentrations.

With respect to stringency conditions for hybridization, it is well known in the art that numerous equivalent conditions can be employed to establish a particular stringency by varying, for example, the following factors: the length and nature of the sequences, base composition of the various sequences, concentrations of salts and other

- 5 hybridization solution components, the presence or absence of blocking agents in the hybridization solutions (e.g., dextran sulfate, and polyethylene glycol), hybridization reaction temperature and time parameters, as well as, varying wash conditions. The selection of a particular set of hybridization conditions is selected following standard methods in the art (see, for example, Sambrook, et al., Molecular Cloning: A Laboratory
10 Manual, Second Edition, (1989) Cold Spring Harbor, N.Y.).

- "Cleavage" refers to the breakage of the covalent backbone of a DNA molecule. Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible, and double-stranded cleavage can occur as a result
15 of two distinct single-stranded cleavage events. DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, fusion polypeptides are used for targeted double-stranded DNA cleavage.

- A "cleavage domain" comprises one or more polypeptide sequences which possesses catalytic activity for DNA cleavage. A cleavage domain can be contained in a
20 single polypeptide chain or cleavage activity can result from the association of two (or more) polypeptides.

A "cleavage half-domain" is a polypeptide sequence which, in conjunction with a second polypeptide (either identical or different) forms a complex having cleavage activity.

- 25 "Chromatin" is the nucleoprotein structure comprising the cellular genome. Cellular chromatin comprises nucleic acid, primarily DNA, and protein, including histones and non-histone chromosomal proteins. The majority of eukaryotic cellular chromatin exists in the form of nucleosomes, wherein a nucleosome core comprises approximately 150 base pairs of DNA associated with an octamer comprising two each of
30 histones H2A, H2B, H3 and H4; and linker DNA (of variable length depending on the organism) extends between nucleosome cores. A molecule of histone H1 is generally

associated with the linker DNA. For the purposes of the present disclosure, the term "chromatin" is meant to encompass all types of cellular nucleoprotein, both prokaryotic and eukaryotic. Cellular chromatin includes both chromosomal and episomal chromatin.

5 A "chromosome," is a chromatin complex comprising all or a portion of the genome of a cell. The genome of a cell is often characterized by its karyotype, which is the collection of all the chromosomes that comprise the genome of the cell. The genome of a cell can comprise one or more chromosomes.

10 An "episome" is a replicating nucleic acid, nucleoprotein complex or other structure comprising a nucleic acid that is not part of the chromosomal karyotype of a cell. Examples of episomes include plasmids and certain viral genomes.

15 An "accessible region" is a site in cellular chromatin in which a target site present in the nucleic acid can be bound by an exogenous molecule which recognizes the target site. Without wishing to be bound by any particular theory, it is believed that an accessible region is one that is not packaged into a nucleosomal structure. The distinct structure of an accessible region can often be detected by its sensitivity to chemical and enzymatic probes, for example, nucleases.

20 A "target site" or "target sequence" is a nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule will bind, provided sufficient conditions for binding exist. For example, the sequence 5'-GAATTC-3' is a target site for the Eco RI restriction endonuclease.

25 An "exogenous" molecule is a molecule that is not normally present in a cell, but can be introduced into a cell by one or more genetic, biochemical or other methods. "Normal presence in the cell" is determined with respect to the particular developmental stage and environmental conditions of the cell. Thus, for example, a molecule that is present only during embryonic development of muscle is an exogenous molecule with respect to an adult muscle cell. Similarly, a molecule induced by heat shock is an exogenous molecule with respect to a non-heat-shocked cell. An exogenous molecule can comprise, for example, a functioning version of a malfunctioning endogenous molecule or a malfunctioning version of a normally-functioning endogenous molecule.

30 An exogenous molecule can be, among other things, a small molecule, such as is generated by a combinatorial chemistry process, or a macromolecule such as a protein,

nucleic acid, carbohydrate, lipid, glycoprotein, lipoprotein, polysaccharide, any modified derivative of the above molecules, or any complex comprising one or more of the above molecules. Nucleic acids include DNA and RNA, can be single- or double-stranded; can be linear, branched or circular; and can be of any length. Nucleic acids include those capable of forming duplexes, as well as triplex-forming nucleic acids. See, for example, U.S. Patent Nos. 5,176,996 and 5,422,251. Proteins include, but are not limited to, DNA-binding proteins, transcription factors, chromatin remodeling factors, methylated DNA binding proteins, polymerases, methylases, demethylases, acetylases, deacetylases, kinases, phosphatases, integrases, recombinases, ligases, topoisomerases, gyrases and helicases.

An exogenous molecule can be the same type of molecule as an endogenous molecule, *e.g.*, an exogenous protein or nucleic acid. For example, an exogenous nucleic acid can comprise an infecting viral genome, a plasmid or episome introduced into a cell, or a chromosome that is not normally present in the cell. Methods for the introduction of exogenous molecules into cells are known to those of skill in the art and include, but are not limited to, lipid-mediated transfer (*i.e.*, liposomes, including neutral and cationic lipids), electroporation, direct injection, cell fusion, particle bombardment, calcium phosphate co-precipitation, DEAE-dextran-mediated transfer and viral vector-mediated transfer.

By contrast, an "endogenous" molecule is one that is normally present in a particular cell at a particular developmental stage under particular environmental conditions. For example, an endogenous nucleic acid can comprise a chromosome, the genome of a mitochondrion, chloroplast or other organelle, or a naturally-occurring episomal nucleic acid. Additional endogenous molecules can include proteins, for example, transcription factors and enzymes.

A "fusion" molecule is a molecule in which two or more subunit molecules are linked, preferably covalently. The subunit molecules can be the same chemical type of molecule, or can be different chemical types of molecules. Examples of the first type of fusion molecule include, but are not limited to, fusion proteins (for example, a fusion between a ZFP DNA-binding domain and a cleavage domain) and fusion nucleic acids (for example, a nucleic acid encoding the fusion protein described *supra*). Examples of

the second type of fusion molecule include, but are not limited to, a fusion between a triplex-forming nucleic acid and a polypeptide, and a fusion between a minor groove binder and a nucleic acid.

Expression of a fusion protein in a cell can result from delivery of the fusion protein to the cell or by delivery of a polynucleotide encoding the fusion protein to a cell, wherein the polynucleotide is transcribed, and the transcript is translated, to generate the fusion protein. Trans-splicing, polypeptide cleavage and polypeptide ligation can also be involved in expression of a protein in a cell. Methods for polynucleotide and polypeptide delivery to cells are presented elsewhere in this disclosure.

A "gene," for the purposes of the present disclosure, includes a DNA region encoding a gene product (see *infra*), as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

"Gene expression" refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (e.g., mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of a mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

"Modulation" of gene expression refers to a change in the activity of a gene. Modulation of expression can include, but is not limited to, gene activation and gene repression.

"Eucaryotic" cells include, but are not limited to, fungal cells (such as yeast), plant cells, animal cells, mammalian cells and human cells.

A "region of interest" is any region of cellular chromatin, such as, for example, a gene or a non-coding sequence within or adjacent to a gene, in which it is desirable to bind an exogenous molecule. Binding can be for the purposes of targeted DNA cleavage

and/or targeted recombination. A region of interest can be present in a chromosome, an episome, an organellar genome (e.g., mitochondrial, chloroplast), or an infecting viral genome, for example. A region of interest can be within the coding region of a gene, within transcribed non-coding regions such as, for example, leader sequences, trailer sequences or introns, or within non-transcribed regions, either upstream or downstream of the coding region. A region of interest can be as small as a single nucleotide pair or up to 2,000 nucleotide pairs in length, or any integral value of nucleotide pairs.

The terms "operative linkage" and "operatively linked" (or "operably linked") are used interchangeably with reference to a juxtaposition of two or more components (such as sequence elements), in which the components are arranged such that both components function normally and allow the possibility that at least one of the components can mediate a function that is exerted upon at least one of the other components. By way of illustration, a transcriptional regulatory sequence, such as a promoter, is operatively linked to a coding sequence if the transcriptional regulatory sequence controls the level of transcription of the coding sequence in response to the presence or absence of one or more transcriptional regulatory factors. A transcriptional regulatory sequence is generally operatively linked in *cis* with a coding sequence, but need not be directly adjacent to it. For example, an enhancer is a transcriptional regulatory sequence that is operatively linked to a coding sequence, even though they are not contiguous.

With respect to fusion polypeptides, the term "operatively linked" can refer to the fact that each of the components performs the same function in linkage to the other component as it would if it were not so linked. For example, with respect to a fusion polypeptide in which a ZFP DNA-binding domain is fused to a cleavage domain, the ZFP DNA-binding domain and the cleavage domain are in operative linkage if, in the fusion polypeptide, the ZFP DNA-binding domain portion is able to bind its target site and/or its binding site, while the cleavage domain is able to cleave DNA in the vicinity of the target site.

A "functional fragment" of a protein, polypeptide or nucleic acid is a protein, polypeptide or nucleic acid whose sequence is not identical to the full-length protein, polypeptide or nucleic acid, yet retains the same function as the full-length protein, polypeptide or nucleic acid. A functional fragment can possess more, fewer, or the same

number of residues as the corresponding native molecule, and/or can contain one or more amino acid or nucleotide substitutions. Methods for determining the function of a nucleic acid (*e.g.*, coding function, ability to hybridize to another nucleic acid) are well-known in the art. Similarly, methods for determining protein function are well-known.

- 5 For example, the DNA-binding function of a polypeptide can be determined, for example, by filter-binding, electrophoretic mobility-shift, or immunoprecipitation assays. DNA cleavage can be assayed by gel electrophoresis. See Ausubel *et al.*, *supra*. The ability of a protein to interact with another protein can be determined, for example, by co-immunoprecipitation, two-hybrid assays or complementation, both genetic and
- 10 biochemical. See, for example, Fields *et al.* (1989) *Nature* 340:245-246; U.S. Patent No. 5,585,245 and PCT WO 98/44350.

Target sites

- The disclosed methods and compositions include fusion proteins comprising a
- 15 cleavage domain (or a cleavage half-domain) and a zinc finger domain, in which the zinc finger domain, by binding to a sequence in cellular chromatin (*e.g.*, a target site or a binding site), directs the activity of the cleavage domain (or cleavage half-domain) to the vicinity of the sequence and, hence, induces cleavage in the vicinity of the target sequence. As set forth elsewhere in this disclosure, a zinc finger domain can be
- 20 engineered to bind to virtually any desired sequence. Accordingly, after identifying a region of interest containing a sequence at which cleavage or recombination is desired, one or more zinc finger binding domains can be engineered to bind to one or more sequences in the region of interest. Expression of a fusion protein comprising a zinc finger binding domain and a cleavage domain (or of two fusion proteins, each comprising
- 25 a zinc finger binding domain and a cleavage half-domain), in a cell, effects cleavage in the region of interest.

- Selection of a sequence in cellular chromatin for binding by a zinc finger domain (*e.g.*, a target site) can be accomplished, for example, according to the methods disclosed in co-owned US Patent No. 6,453,242 (Sept. 17, 2002), which also discloses methods for
- 30 designing ZFPs to bind to a selected sequence. Additional non-limiting exemplary methods for target site selection and ZFP design are disclosed in co-owned US Patent

Application entitled "Selection of Target Sequences for Zinc Finger Proteins" (Reference No. S37-PR; Attorney Docket No. 019496-006900US) filed even date herewith. It will be clear to those skilled in the art that simple visual inspection of a nucleotide sequence can also be used for selection of a target site. Accordingly, any means for target site selection can be used in the claimed methods.

Target sites are generally composed of a plurality of adjacent target subsites. A target subsite refers to the sequence (usually either a nucleotide triplet, or a nucleotide quadruplet that can overlap by one nucleotide with an adjacent quadruplet) bound by an individual zinc finger. See, for example, WO 02/077227. If the strand with which a zinc finger protein makes most contacts is designated the target strand "primary recognition strand," or "primary contact strand," some zinc finger proteins bind to a three base triplet in the target strand and a fourth base on the non-target strand. A target site generally has a length of at least 9 nucleotides and, accordingly, is bound by a zinc finger binding domain comprising at least three zinc fingers. However binding of, for example, a 4-finger binding domain to a 12-nucleotide target site, a 5-finger binding domain to a 15-nucleotide target site or a 6-finger binding domain to an 18-nucleotide target site, is also possible. As will be apparent, binding of larger binding domains (*e.g.*, 7-, 8-, 9-finger and more) to longer target sites is also possible.

It is not necessary for a target site to be a multiple of three nucleotides. For example, in cases in which cross-strand interactions occur (see, *e.g.*, US Patent 6,453,242 and WO 02/077227), one or more of the individual zinc fingers of a multi-finger binding domain can bind to overlapping quadruplet subsites. As a result, a three-finger protein can bind a 10-nucleotide sequence, wherein the tenth nucleotide is part of a quadruplet bound by a terminal finger, a four-finger protein can bind a 13-nucleotide sequence, wherein the thirteenth nucleotide is part of a quadruplet bound by a terminal finger, *etc.*

The length and nature of amino acid linker sequences between individual zinc fingers in a multi-finger binding domain also affects binding to a target sequence. For example, the presence of a so-called "non-canonical linker," "long linker" or "structured linker" between adjacent zinc fingers in a multi-finger binding domain can allow those fingers to bind subsites which are not immediately adjacent. Non-limiting examples of such linkers are described, for example, in US Patent No. 6,479,626 and WO 01/53480.

Accordingly, one or more subsites, in a target site for a zinc finger binding domain, can be separated from each other by 1, 2, 3, 4, 5 or more nucleotides. To provide but one example, a four-finger binding domain can bind to a 13-nucleotide target site comprising, in sequence, two contiguous 3-nucleotide subsites, an intervening nucleotide, and two contiguous triplet subsites.

Distance between sequences (e.g., target sites) refers to the number of nucleotides or nucleotide pairs intervening between two sequences, as measured from the edges of the sequences nearest each other.

Zinc finger binding domains

A zinc finger binding domain comprises one or more zinc fingers. Miller *et al.* (1985) *EMBO J.* **4**:1609-1614; Rhodes (1993) *Scientific American* **Feb.**:56-65; US Patent No. 6,453,242. Typically, a single zinc finger domain is about 30 amino acids in length. Structural studies have demonstrated that each zinc finger domain (motif) contains two beta sheets (held in a beta turn which contains the two invariant cysteine residues) and an alpha helix (containing the two invariant histidine residues), which are held in a particular conformation through coordination of a zinc atom by the two cysteines and the two histidines.

Zinc fingers include both canonical C_2H_2 zinc fingers (*i.e.*, those in which the zinc ion is coordinated by two cysteine and two histidine residues) and non-canonical zinc fingers such as, for example, C_3H zinc fingers (those in which the zinc ion is coordinated by three cysteine residues and one histidine residue) and C_4 zinc fingers (those in which the zinc ion is coordinated by four cysteine residues). See also WO 02/057293.

Zinc finger binding domains can be engineered to bind to a sequence of choice. See, for example, Beerli *et al.* (2002) *Nature Biotechnol.* **20**:135-141; Pabo *et al.* (2001) *Ann. Rev. Biochem.* **70**:313-340; Isalan *et al.* (2001) *Nature Biotechnol.* **19**:656-660; Segal *et al.* (2001) *Curr. Opin. Biotechnol.* **12**:632-637; Choo *et al.* (2000) *Curr. Opin. Struct. Biol.* **10**:411-416. An engineered zinc finger binding domain can have a novel binding specificity, compared to a naturally-occurring zinc finger protein. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising triplet (or quadruplet)

nucleotide sequences and individual zinc finger amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence. See, for example, co-owned U.S. Patents 6,453,242 and 6,534,261.

5 Exemplary selection methods, including phage display and two-hybrid systems, are disclosed in US Patents 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; WO 01/88197 and GB 2,338,237.

10 Enhancement of binding specificity for zinc finger binding domains has been described, for example, in co-owned WO 02/077227.

Since an individual zinc finger binds to a three-nucleotide (*i.e.*, triplet) sequence (or a four-nucleotide sequence which can overlap, by one nucleotide, with the four-nucleotide binding site of an adjacent zinc finger), the length of a sequence to which a zinc finger binding domain is engineered to bind (*e.g.*, a target sequence) will determine
15 the number of zinc fingers in an engineered zinc finger binding domain. For example, for ZFPs in which the finger motifs do not bind to overlapping subsites, a six-nucleotide target sequence is bound by a two-finger binding domain; a nine-nucleotide target sequence is bound by a three-finger binding domain, *etc.* As noted herein, binding sites for individual zinc fingers (*i.e.*, subsites) in a target site need not be contiguous, but can
20 be separated by one or several nucleotides, depending on the length and nature of the amino acids sequences between the zinc fingers (*i.e.*, the inter-finger linkers) in a multi-finger binding domain.

In a multi-finger zinc finger binding domain, adjacent zinc fingers can be separated by amino acid linker sequences of approximately 5 amino acids (so-called
25 "canonical" inter-finger linkers) or, alternatively, by one or more non-canonical linkers. See, *e.g.*, co-owned US Patent Nos. 6,453,242 and 6,534,261. For engineered zinc finger binding domains comprising more than three fingers, insertion of longer ("non-canonical") inter-finger linkers between certain of the zinc fingers may be preferred as it may increase the affinity and/or specificity of binding by the binding domain. See, for
30 example, U.S. Patent No. 6,479,626 and WO 01/53480. Accordingly, multi-finger zinc finger binding domains can also be characterized with respect to the presence and

location of non-canonical inter-finger linkers. For example, a six-finger zinc finger binding domain comprising three fingers (joined by two canonical inter-finger linkers), a long linker and three additional fingers (joined by two canonical inter-finger linkers) is denoted a 2x3 configuration. Similarly, a binding domain comprising two fingers (with a canonical linker therebetween), a long linker and two additional fingers (joined by a canonical linker) is denoted a 2x2 protein. A protein comprising three two-finger units (in each of which the two fingers are joined by a canonical linker), and in which each two-finger unit is joined to the adjacent two finger unit by a long linker, is referred to as a 3x2 protein.

The presence of a long or non-canonical inter-finger linker between two adjacent zinc fingers in a multi-finger binding domain often allows the two fingers to bind to subsites which are not immediately contiguous in the target sequence. Accordingly, there can be gaps of one or more nucleotides between subsites in a target site; *i.e.*, a target site can contain one or more nucleotides that are not contacted by a zinc finger. For example, a 2x2 zinc finger binding domain can bind to two six-nucleotide sequences separated by one nucleotide, *i.e.*, it binds to a 13-nucleotide target site. See also Moore *et al.* (2001a) Proc. Natl. Acad. Sci. USA 98:1432-1436; Moore *et al.* (2001b) Proc. Natl. Acad. Sci. USA 98:1437-1441 and WO 01/53480.

As mentioned previously, a target subsite is a three- or four-nucleotide sequence that is bound by a single zinc finger. For certain purposes, a two-finger unit is denoted a binding module. A binding module can be obtained by, for example, selecting for two adjacent fingers in the context of a multi-finger protein (generally three fingers) which bind a particular six-nucleotide target sequence. Alternatively, modules can be constructed by assembly of individual zinc fingers. See also WO 98/53057; WO 01/53480 and co-owned US Patent Application entitled "Selection of Target Sequences for Zinc Finger Proteins" (Reference No. S37-PR, Attorney Docket No. 019496-006900US), filed even date herewith.

Cleavage domains

The cleavage domain portion of the fusion proteins disclosed herein can be obtained from any endo- or exonuclease. Exemplary endonucleases from which a

cleavage domain can be derived include, but are not limited to, restriction endonucleases and homing endonucleases. *See, for example, 2002-2003 Catalogue, New England Biolabs, Beverly, MA; and Belfort et al. (1997) Nucleic Acids Res. 25:3379-3388.*

Additional enzymes which cleave DNA are known (*e.g.*, S1 Nuclease; mung bean nuclease; pancreatic DNase I; micrococcal nuclease; yeast HO endonuclease; *see also* Linn *et al.* (eds.) *Nucleases*, Cold Spring Harbor Laboratory Press, 1993). One or more of these enzymes (or functional fragments thereof) can be used as a source of cleavage domains and cleavage half-domains.

Similarly, a cleavage half-domain (*e.g.*, fusion proteins comprising a zinc finger binding domain and a cleavage half-domain) can be derived from any nuclease or portion thereof, as set forth above, that requires dimerization for cleavage activity. In general, two fusion proteins are required for cleavage if the fusion proteins comprise cleavage half-domains. The two cleavage half-domains can be derived from the same endonuclease (or functional fragments thereof), or each cleavage half-domain can be derived from a different endonuclease (or functional fragments thereof). In addition, the target sites for the two fusion proteins are preferably disposed, with respect to each other, such that binding of the two fusion proteins places the cleavage half-domains in a spatial orientation to each other that allows the cleavage half-domains to form a functional cleavage domain, *e.g.*, by dimerizing. Thus, in certain embodiments, the near edges of the target sites are separated by 5-8 nucleotides or by 15-18 nucleotides. However any integral number of nucleotides or nucleotide pairs can intervene between two target sites (*e.g.*, from 2 to 50 nucleotides or more). In general, the point of cleavage lies between the target sites.

In general, if two fusion proteins are used, each comprising a cleavage half-domain, the primary contact strand for the zinc finger portion of each fusion protein will be on a different DNA strands and in opposite orientation. That is, for a pair of ZFP/cleavage half-domain fusions, the target sequences are on opposite strands and the two proteins bind in opposite orientations.

Restriction endonucleases (restriction enzymes) are present in many species and are capable of sequence-specific binding to DNA (at a recognition site), and cleaving DNA at or near the site of binding. Certain restriction enzymes (*e.g.*, Type IIS) cleave

DNA at sites removed from the recognition site and have separable binding and cleavage domains. For example, the Type IIS enzyme *Fok I* catalyzes double-stranded cleavage of DNA, at 9 nucleotides from its recognition site on one strand and 13 nucleotides from its recognition site on the other. See, for example, US Patents 5,356,802; 5,436,150 and 5,487,994; as well as Li *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89**:4275-4279; Li *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90**:2764-2768; Kim *et al.* (1994a) *Proc. Natl. Acad. Sci. USA* **91**:883-887; Kim *et al.* (1994b) *J. Biol. Chem.* **269**:31,978-31,982. Thus, in one embodiment, fusion proteins comprise the cleavage domain (or cleavage half-domain) from at least one Type IIS restriction enzyme and one or more zinc finger binding domains, which may or may not be engineered.

An exemplary Type IIS restriction enzyme, whose cleavage domain is separable from the binding domain, is *Fok I*. This particular enzyme is active as a dimer. Bitinaite *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**: 10,570-10,575. Accordingly, for the purposes of the present disclosure, the portion of the *Fok I* enzyme used in the disclosed fusion proteins is considered a cleavage half-domain. Thus, for targeted double-stranded cleavage and/or targeted replacement of cellular sequences using zinc finger-*Fok I* fusions, two fusion proteins are used to reconstitute a catalytically active cleavage domain. Parameters for targeted cleavage and targeted sequence replacement using zinc finger-*Fok I* fusions are provided elsewhere in this disclosure.

Exemplary Type IIS restriction enzymes are listed in Table 1. Additional restriction enzymes also contain separable binding and cleavage domains, and these are contemplated by the present disclosure. See, for example, Roberts *et al.* (2003) *Nucleic Acids Res.* **31**:418-420.

Table 1: Some Type IIS Restriction Enzymes

Aar I	BsrB I	SspD5 I
Ace III	BsrD I	Sth132 I
Aci I	BstF5 I	Sts I
Alo I	Btr I	TspDT I
Bae I	Bts I	TspGW I
Bbr7 I	Cdi I	Tth111 II

	Bbv I	CjeP I	UbaP I
	Bbv II	Drd II	Bsa I
	BbvC I	Eci I	BsmB I
	Bcc I	Eco31 I	
5	Bce83 I	Eco57 I	
	BceA I	Eco57M I	
	Bcef I	Esp3 I	
	Bcg I	Fau I	
	BciV I	Fin I	
10	Bfi I	Fok I	
	Bin I	Gdi II	
	Bmg I	Gsu I	
	Bpu10 I	Hga I	
	BsaX I	Hin4 II	
15	Bsb I	Hph I	
	BscA I	Ksp632 I	
	BscG I	Mbo II	
	BseR I	Mly I	
	BseY I	Mme I	
20	Bsi I	Mnl I	
	Bsm I	Pfl1108 I	
	BsmA I	Ple I	
	BsmF I	Ppi I	
	Bsp24 I	Psr I	
25	BspG I	RleA I	
	BspM I	Sap I	
	BspNC I	SfaN I	
	Bsr I	Sim I	

Zinc finger domain-cleavage domain fusions

Methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art. For example, methods for the design and construction of fusion protein comprising zinc finger proteins (and polynucleotides encoding same) are described in co-owned US Patents 6,453,242 and 6,534,261. In certain embodiments, polynucleotides encoding such fusion proteins are constructed. These polynucleotides can be inserted into a vector and the vector can be introduced into a cell (see below for additional disclosure regarding vectors and methods for introducing polynucleotides into cells).

In certain embodiments of the methods described herein, a fusion protein comprises a zinc finger binding domain and a cleavage half-domain from the *Fok I* restriction enzyme, and two such fusion proteins are expressed in a cell. Expression of two fusion proteins in a cell can result from delivery of the two proteins to the cell; delivery of one protein and one nucleic acid encoding one of the proteins to the cell; delivery of two nucleic acids, each encoding one of the proteins, to the cell; or by delivery of a single nucleic acid, encoding both proteins, to the cell. In additional embodiments, a fusion protein comprises a single polypeptide chain comprising two cleavage half domains and a zinc finger binding domain. In this case, a single fusion protein is expressed in a cell and, without wishing to be bound by theory, is believed to cleave DNA as a result of formation of an intramolecular dimer of the cleavage half-domains.

In general, the components of the fusion proteins (e.g. ZFP-*Fok I* fusions) are arranged such that the zinc finger domain is nearest the amino terminus of the fusion protein, and the cleavage half-domain is nearest the carboxy-terminus. This mirrors the relative orientation of the cleavage domain in naturally-occurring dimerizing cleavage domains such as those derived from the *Fok I* enzyme, in which the DNA-binding domain is nearest the amino terminus and the cleavage half-domain is nearest the carboxy terminus.

In the disclosed fusion proteins, the amino acid sequence between the zinc finger binding domain (which is delimited by the N-terminal most of the two conserved cysteine residues and the C-terminal-most of the two conserved histidine residues) and the

cleavage domain (or half-domain) is denoted the "ZC linker." The ZC linker is to be distinguished from the inter-finger linkers discussed above. For instance, in a ZFP-*Fok I* fusion protein (in which the components are arranged: N terminus-zinc finger binding domain-*Fok I* cleavage half domain-C terminus), the ZC linker is located between the second histidine residue of the C-terminal-most zinc finger and the N-terminal-most amino acid residue of the cleavage half-domain (which is generally glutamine (Q) in the sequence QLV). The ZC linker can be any amino acid sequence. To obtain optimal cleavage, the length of the linker and the distance between the target sites (binding sites) are interrelated. See, for example, Smith *et al.* (2000) *Nucleic Acids Res.* **28**:3361-3369; Bibikova *et al.* (2001) *Mol. Cell. Biol.* **21**:289-297, noting that their notation for linker length differs from that given here. For example, for ZFP-*Fok I* fusions having a ZC linker length of four amino acids (as defined herein), optimal cleavage occurs when the binding sites for the fusion proteins are located 6 or 16 nucleotides apart (as measured from the near edge of each binding site).

Methods for targeted cleavage

The disclosed methods and compositions can be used to cleave DNA at a region of interest in cellular chromatin (*e.g.*, at a desired or predetermined site in a genome, for example, in a gene, either mutant or wild-type). For such targeted DNA cleavage, a zinc finger binding domain is engineered to bind a target site at or near the predetermined cleavage site, and a fusion protein comprising the engineered zinc finger binding domain and a cleavage domain is expressed in a cell. Upon binding to the target site, the DNA is cleaved. Alternatively, two fusion proteins, each comprising a zinc finger binding domain and a cleavage half-domain, are expressed in a cell, and bind to target sites which are juxtaposed in such a way that a functional cleavage domain is reconstituted. One or both of the zinc finger binding domains can be engineered.

For targeted cleavage using a zinc finger binding domain-cleavage domain fusion polypeptide, the binding site can encompass the cleavage site, or the near edge of the binding site can be 1, 2, 3, 4, 5, 10, 25, 50 or more nucleotides (or any integral value between 1 and 50 nucleotides) from the cleavage site. The exact location of the binding site, with respect to the cleavage site, will depend upon the particular cleavage domain,

and the length of the ZC linker. For methods in which two fusion polypeptides, each comprising a zinc finger binding domain and a cleavage half-domain, are used, the binding sites generally straddle the cleavage site. Thus the near edge of the first binding site can be 1, 2, 3, 4, 5, 10, 25 or more nucleotides (or any integral value between 1 and 50 nucleotides) on one side of the cleavage site, and the near edge of the second binding site can be 1, 2, 3, 4, 5, 10, 25 or more nucleotides (or any integral value between 1 and 50 nucleotides) on the other side of the cleavage site. Methods for mapping cleavage sites *in vitro* and *in vivo* are known to those of skill in the art.

Thus, the methods described herein can employ an engineered zinc finger binding domain fused to a cleavage domain. In these cases, the binding domain is engineered to bind to a target sequence, at or near which cleavage is desired. The fusion protein, or a polynucleotide encoding same, is introduced into a cell. Once introduced into, or expressed in, the cell, the fusion protein binds to the target sequence and cleaves at or near the target sequence. The exact site of cleavage depends on the nature of the cleavage domain and/or the presence and/or nature of linker sequences between the binding and cleavage domains. In cases where two fusion proteins, each comprising a cleavage half-domain, are used, optimal levels of cleavage can also depend on the distance between the binding sites of the two fusion proteins. See, for example, Smith *et al.* (2000) *Nucleic Acids Res.* **28**:3361-3369; Bibikova *et al.* (2001) *Mol. Cell. Biol.* **21**:289-297.

For ZFP-*FokI* fusion nucleases, the length of the linker between the ZFP and the *FokI* cleavage half-domain (*i.e.*, the ZC linker) can influence cleavage efficiency. In one experimental system utilizing a ZFP-*FokI* fusion with a ZC linker of 4 amino acid residues, optimal cleavage was obtained when the near edges of the binding sites for two ZFP-*FokI* nucleases were separated by 6 base pairs. This particular fusion nuclease comprised the following amino acid sequence between the zinc finger portion and the nuclease half-domain:

HQRTHQNKKQLV (SEQ ID NO:**)

in which the two conserved histidines in the C-terminal portion of the zinc finger and the first three residues in the *FokI* cleavage half-domain are underlined. Accordingly, the linker sequence in this construct is QNKK. Bibikova *et al.* (2001) *Mol. Cell. Biol.*

21:289-297. The present inventors have constructed a number of ZFP-*FokI* fusion nucleases having a variety of ZC linker lengths and sequences, and analyzed the cleavage efficiencies of these nucleases on a series of substrates having different distances between the ZFP binding sites. See Example 4.

5 In certain embodiments, the cleavage domain comprises two cleavage half-domains, both of which are part of a single polypeptide comprising a binding domain, a first cleavage half-domain and a second cleavage half-domain. The cleavage half-domains can have the same amino acid sequence or different amino acid sequences, so long as they function to cleave the DNA.

10 Cleavage half-domains may also be provided in separate molecules. For example, two fusion polypeptides may be introduced into a cell, wherein each polypeptide comprises a binding domain and a cleavage half-domain. The cleavage half-domains can have the same amino acid sequence or different amino acid sequences, so long as they function to cleave the DNA.. Further, the binding domains bind to target sequences
15 which are typically disposed in such a way that, upon binding of the fusion polypeptides, the two cleavage half-domains are presented in a spatial orientation to each other that allows reconstitution of a cleavage domain (*e.g.*, by dimerization of the half-domains), thereby positioning the half-domains relative to each other to form a functional cleavage domain, resulting in cleavage of cellular chromatin in a region of interest. Generally,
20 cleavage by the reconstituted cleavage domain occurs at a site located between the two target sequences. One or both of the proteins can be engineered to bind to its target site.

The two fusion proteins can bind in the region of interest in the same or opposite polarity, and their binding sites (*i.e.*, target sites) can be separated by any number of nucleotides, *e.g.*, from 0 to 200 nucleotides or any integral value therebetween. In certain
25 embodiments, the binding sites for two fusion proteins, each comprising a zinc finger binding domain and a cleavage half-domain, can be located between 5 and 18 nucleotides apart, for example, 5-8 nucleotides apart, or 15-18 nucleotides apart, or 6 nucleotides apart, or 16 nucleotides apart, as measured from the edge of each binding site nearest the other binding site, and cleavage occurs between the binding sites.

30 The site at which the DNA is cleaved generally lies between the binding sites for the two fusion proteins. Double-strand breakage of DNA often results from two single-

strand breaks, or “nicks,” offset by 1, 2, 3, 4, 5, 6 or more nucleotides, (for example, cleavage of double-stranded DNA by native *Fok* I results from single-strand breaks offset by 4 nucleotides). Thus, cleavage does not necessarily occur at exactly opposite sites on both DNA strands. In addition, the structure of the fusion proteins and the distance
5 between the target sites can influence whether cleavage occurs adjacent a single nucleotide pair, or whether cleavage occurs at several sites. However, for many applications, including targeted recombination (see *infra*) cleavage within a range of nucleotides is generally sufficient, and cleavage between particular base pairs is not required.

10 As noted above, the fusion protein(s) can be introduced as polypeptides and/or polynucleotides. For example, two polynucleotides, each comprising sequences encoding one of the aforementioned polypeptides, can be introduced into a cell, and when the polypeptides are expressed and each binds to its target sequence, cleavage occurs at or near the target sequence. Alternatively, a single polynucleotide comprising sequences
15 encoding both fusion polypeptides is introduced into a cell.

To enhance cleavage specificity, additional compositions may also be employed in the methods described herein. For example, single cleavage half-domains can exhibit limited double-stranded cleavage activity. In methods in which two fusion proteins, each containing a three-finger zinc finger domain and a cleavage half-domain, are introduced
20 into the cell, either protein specifies an approximately 9-nucleotide target site. Although the aggregate target sequence of 18 nucleotides is likely to be unique in a mammalian genome, any given 9-nucleotide target site occurs, on average, approximately 23,000 times in the human genome. Thus, non-specific cleavage, due to the activity of a single half-domain, may occur. Accordingly, the methods described herein contemplate the use
25 of a dominant-negative mutant of a cleavage half-domain such as *Fok* I (or a nucleic acid encoding same) that is expressed in a cell along with the two fusion proteins. The dominant-negative mutant is capable of dimerizing but is unable to cleave, and also blocks the cleavage activity of a half-domain to which it is dimerized. By providing the dominant-negative mutant in molar excess to the fusion proteins, only regions in which
30 both fusion proteins are bound will have a high enough local concentration of functional cleavage half-domains for dimerization and cleavage to occur. At sites where only one of

the two fusion proteins are bound, its cleavage half-domain forms a dimer with the dominant negative mutant half-domain, and undesirable, non-specific cleavage does not occur.

Three catalytic amino acid residues in the *Fok I* cleavage half-domain have been identified: Asp 450, Asp 467 and Lys 469. Bitinaite *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**: 10,570-10,575. Thus, one or more mutations at one of these residues can be used to generate a dominant negative mutation. Further, many of the catalytic amino acid residues of other Type IIS endonucleases are known and/or can be determined, for example, by alignment with *Fok I* sequences and/or by generation and testing of mutants for catalytic activity.

Dimerization domain mutations in the cleavage half-domain

Methods for targeted cleavage which involve the use of fusions between a ZFP and a cleavage half-domain (such as, *e.g.*, a ZFP/*FokI* fusion) require the use of two such fusion molecules, each generally directed to a distinct target sequence. Target sequences for the two fusion proteins can be chosen so that targeted cleavage is directed to a unique site in a genome, as discussed above. A potential source of reduced cleavage specificity could result from homodimerization of one of the two ZFP/cleavage half-domain fusions. This might occur, for example, due to the presence, in a genome, of inverted repeats of the target sequences for one of the two ZFP/cleavage half-domain fusions, located so as to allow two copies of the same fusion protein to bind with an orientation and spacing that allows formation of a functional dimer.

One approach for reducing the probability of this type of aberrant cleavage at sequences other than the intended target site involves generating variants of the cleavage half-domain that minimize or prevent homodimerization. Preferably, one or more amino acids in the region of the half-domain involved in its dimerization are altered. In the crystal structure of the *FokI* protein dimer, the structure of the cleavage half-domains is reported to be similar to the arrangement of the cleavage half-domains during cleavage of DNA by *FokI*. Wah *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**:10564-10569. This structure indicates that amino acid residues at positions 483 and 487 play a key role in the dimerization of the *FokI* cleavage half-domains. The structure also indicates that amino

acid residues at positions 446, 447, 479, 483, 484, 486, 487, 490, 491, 496, 498, 499, 500, 531, 534, 537, and 538 are all close enough to the dimerization interface to influence dimerization. Accordingly, amino acid sequence alterations at one or more of the aforementioned positions will likely alter the dimerization properties of the cleavage half-domain. Such changes can be introduced, for example, by constructing a library containing (or encoding) different amino acid residues at these positions and selecting variants with the desired properties, or by rationally designing individual mutants. In addition to preventing homodimerization, it is also possible that some of these mutations may increase the cleavage efficiency above that obtained with two wild-type cleavage half-domains.

Accordingly, alteration of a *FokI* cleavage half-domain at any amino acid residue which affects dimerization can be used to prevent one of a pair of ZFP/*FokI* fusions from undergoing homodimerization which can lead to cleavage at undesired sequences. Thus, for targeted cleavage using a pair of ZFP/*FokI* fusions, one or both of the fusion proteins can comprise one or more amino acid alterations that inhibit self-dimerization, but allow heterodimerization of the two fusion proteins to occur such that cleavage occurs at the desired target site. In certain embodiments, alterations are present in both fusion proteins, and the alterations have additive effects; *i.e.*, homodimerization of either fusion, leading to aberrant cleavage, is minimized or abolished, while heterodimerization of the two fusion proteins is facilitated compared to that obtained with wild-type cleavage half-domains. See Example 5.

Methods for targeted replacement of genomic sequences and targeted recombination

Also described herein are methods of replacing a genomic sequence (*e.g.*, a region of interest in cellular chromatin) with a homologous non-identical sequence (*i.e.*, targeted recombination). Previous attempts to replace particular sequences have involved contacting a cell with a polynucleotide comprising sequences bearing homology to a chromosomal region (*i.e.*, a donor DNA), followed by selection of cells in which the donor DNA molecule had undergone homologous recombination into the genome. The success rate of these methods is low, due to poor efficiency of homologous

recombination and a high frequency of non-specific insertion of the donor DNA into regions of the genome other than the target site.

The present disclosure provides methods of targeted sequence replacement characterized by a greater efficiency of targeted recombination and a lower frequency of non-specific insertion events. The methods involve making and using engineered zinc finger binding domains fused to cleavage domains (or cleavage half-domains) to make one or more targeted double-stranded breaks in cellular DNA. Because double-stranded breaks in cellular DNA stimulate homologous recombination several thousand-fold in the vicinity of the cleavage site, such targeted cleavage allows for the replacement (via homologous recombination) of sequences at virtually any site in the genome.

In addition to the fusion molecules described herein, targeted replacement of a selected genomic sequence also requires the introduction of the replacement (or donor) sequence. The donor sequence can be introduced into the cell prior to, concurrently with, or subsequent to, expression of the fusion protein(s). The donor polynucleotide contains sufficient homology to a genomic sequence to support homologous recombination between it and the genomic sequence to which it bears homology. Approximately 25, 50, 100 or 200 nucleotides of sequence homology between a donor and a genomic sequence (or any integral value between 10 and 200 nucleotides) will support homologous recombination therebetween. Donor sequences can range in length from 10 to 5,000 nucleotides (or any integral value of nucleotides therebetween) or longer. It will be readily apparent that the donor sequence is typically not identical to the genomic sequence that it replaces. For example, the sequence of the donor polynucleotide can contain one or more single base changes, insertions, deletions, inversions or rearrangements with respect to the genomic sequence, so long as sufficient homology is present to support homologous recombination. Generally, a donor sequence will have at least 50% sequence identity to a genomic sequence with which recombination is desired. In certain embodiments, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or 99.9% sequence identity is present. Any value between 1% and 100% sequence identity can be present, depending upon the length of the donor polynucleotide.

To simplify assays (*e.g.*, hybridization, PCR, restriction enzyme digestion) for determining successful insertion of the donor sequence, certain sequence differences may

be present in the donor sequence as compared to the genomic sequence. Preferably, if located in a coding region, such nucleotide sequence differences will not change the amino acid sequence, or will make silent amino acid changes (*i.e.*, changes which do not affect the structure or function of the protein).

5 The donor polynucleotide can be DNA or RNA, single-stranded or double-stranded and can be introduced into a cell in linear or circular form. If introduced in linear form, the ends of the donor sequence can be protected (*e.g.*, from exonucleolytic degradation) by methods known to those of skill in the art. For example, one or more dideoxynucleotide residues are added to the 3' terminus of a linear molecule and/or self-
10 complementary oligonucleotides are ligated to one or both ends. *See*, for example, Chang *et al.* (1987) *Proc. Natl. Acad. Sci. USA* **84**:4959-4963; Nehls *et al.* (1996) *Science* **272**:886-889. Additional methods for protecting exogenous polynucleotides from degradation include, but are not limited to, addition of terminal amino group(s) and the use of modified internucleotide linkages such as, for example, phosphorothioates,
15 phosphoramidates, and O-methyl ribose or deoxyribose residues. If introduced in circular form, a polynucleotide can be introduced a part of a vector molecule having additional sequences such as, for example, replication origins, promoters and genes encoding antibiotic resistance. Moreover, donor polynucleotides can be introduced as naked nucleic acid, as nucleic acid complexed with an agent such as a liposome or
20 poloxamer, or can be delivered by viruses (*e.g.*, adenovirus, AAV).

Without being bound by one theory, it appears that the presence of a double-stranded break in a cellular sequence, coupled with the presence of an exogenous DNA molecule having homology to a region adjacent to or surrounding the break, activates cellular mechanisms which repair the break by unidirectional transfer of sequence
25 information from the donor molecule into the cellular (*e.g.*, genomic or chromosomal) sequence; *i.e.*, by a processes of homologous recombination. Applicants' methods advantageously combine the powerful targeting capabilities of engineered ZFPs with a cleavage domain (or cleavage half-domain) to specifically target a double-stranded break to the region of the genome at which recombination is desired.

30 The efficiency of insertion of donor sequences by homologous recombination is inversely related to the distance, in the cellular DNA, between the double-stranded break

and the site at which recombination is desired. In other words, higher homologous recombination efficiencies are observed when the double-stranded break is closer to the site at which recombination is desired. In cases in which a precise site of recombination is not predetermined (*e.g.*, the desired recombination event can occur over an interval of genomic sequence), the length and sequence of the donor nucleic acid, together with the site(s) of cleavage, are selected to obtain the desired recombination event. In cases in which the desired event is designed to change the sequence of a single nucleotide pair in a genomic sequence, cellular chromatin is cleaved within 1,000 nucleotides on either side of that nucleotide pair. In certain embodiments, cleavage occurs within 500, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, or 2 nucleotides, or any integral value between 2 and 1,000 nucleotides, on either side of the nucleotide pair whose sequence is to be changed.

As detailed above, the binding sites for two fusion proteins, each comprising a zinc finger binding domain and a cleavage half-domain, can be located 5-8 or 15-18 nucleotides apart, as measured from the edge of each binding site nearest the other binding site, and cleavage occurs between the binding sites. Whether cleavage occurs at a single site or at multiple sites between the binding sites is immaterial, since the cleaved genomic sequences are replaced by the donor sequences. Thus, for efficient alteration of the sequence of a single nucleotide pair by targeted recombination, the midpoint of the region between the binding sites is within 1,000 nucleotides of that nucleotide pair, preferably within 200 nucleotides, or 100 nucleotides, or 50 nucleotides, or 20 nucleotides, or 10 nucleotides, or 5 nucleotide, or 2 nucleotides, or one nucleotide, or at the nucleotide pair of interest.

In certain embodiments, a homologous chromosome can serve as the donor polynucleotide. Thus, for example, correction of a mutation in a heterozygote can be achieved by engineering fusion proteins which bind to and cleave the mutant sequence on one chromosome, but do not cleave the wild-type sequence on the homologous chromosome. The double-stranded break on the mutation-bearing chromosome stimulates a homology-based "gene conversion" process in which the wild-type sequence from the homologous chromosome is copied into the cleaved chromosome, thus restoring two copies of the wild-type sequence.

Methods and compositions are also provided that may enhance levels of targeted recombination including, but not limited to, the use of additional ZFP-functional domain fusions to activate expression of genes involved in homologous recombination, such as, for example, members of the RAD52 epistasis group (*e.g.*, *Rad50*, *Rad51*, *Rad51B*, *Rad51C*, *Rad51D*, *Rad52*, *Rad54*, *Rad54B*, *Mre11*, *XRCC2*, *XRCC3*), genes whose products interact with the aforementioned gene products (*e.g.*, BRCA1, BRCA2) and/or genes in the NBS1 complex. Similarly ZFP-functional domain fusions can be used, in combination with the methods and compositions disclosed herein, to repress expression of genes involved in non-homologous end joining (*e.g.*, Ku70/80, XRCC4, poly(ADP ribose) polymerase). *See*, for example, Yanez *et al.* (1998) *Gene Therapy* 5:149-159; Hoeijmakers (2001) *Nature* 411:366-374; Johnson *et al.* (2001) *Biochem. Soc. Trans.* 29:196-201; Tauchi *et al.* (2002) *Oncogene* 21:8967-8980. Methods for activation and repression of gene expression using fusions between a zinc finger binding domain and a functional domain are disclosed in co-owned US Patent No. 6,534,261. Additional repression methods include the use of antisense oligonucleotides and/or small interfering RNA (siRNA or RNAi) targeted to the sequence of the gene to be repressed.

As an alternative to or, in addition to, activating expression of gene products involved in homologous recombination, fusions of these protein (or functional fragments thereof) with a zinc finger binding domain targeted to the region of interest, can be used to recruit these proteins (recombination proteins) to the region of interest, thereby increasing their local concentration and further stimulating homologous recombination processes. Alternatively, a polypeptide involved in homologous recombination as described above (or a functional fragment thereof) can be part of a triple fusion protein comprising a zinc finger binding domain, a cleavage domain (or cleavage half-domain) and the recombination protein (or functional fragment thereof). Additional proteins involved in gene conversion and recombination-related chromatin remodeling, which can be used in the aforementioned methods and compositions, include histone acetyltransferases (*e.g.*, Esa1p, Tip60), histone methyltransferases (*e.g.*, Dot1p), histone kinases and histone phosphatases.

The p53 protein has been reported to play a central role in repressing homologous recombination (HR). *See*, for example, Valerie *et al.*, (2003) *Oncogene* 22:5792-5812;

Janz, *et al.* (2002) *Oncogene* **21**:5929-5933. For example, the rate of HR in p53-deficient human tumor lines is 10,000-fold greater than in primary human fibroblasts, and there is a 100-fold increase in HR in tumor cells with a non-functional p53 compared to those with functional p53. Mekeel *et al.* (1997) *Oncogene* **14**:1847-1857. In addition,
5 overexpression of p53 dominant negative mutants leads to a 20-fold increase in spontaneous recombination. Bertrand *et al.* (1997) *Oncogene* **14**:1117-1122. Analysis of different p53 mutations has revealed that the roles of p53 in transcriptional transactivation and G1 cell cycle checkpoint control are separable from its involvement in HR. Saintigny *et al.* (1999) *Oncogene* **18**:3553-3563; Boehden *et al.* (2003) *Oncogene*
10 **22**:4111-4117. Accordingly, downregulation of p53 activity can serve to increase the efficiency of targeted homologous recombination using the methods and compositions disclosed herein. Any method for downregulation of p53 activity can be used, including but not limited to cotransfection and overexpression of a p53 dominant negative mutant or targeted repression of p53 gene expression according to methods disclosed, *e.g.*, in co-
15 owned U.S. Patent No. 6,534,261.

Further increases in efficiency of targeted recombination, in cells comprising a zinc finger/nuclease fusion molecule and a donor DNA molecule, are achieved by blocking the cells in the G₂ phase of the cell cycle, when homology-driven repair processes are maximally active. Such arrest can be achieved in a number of ways. For
20 example, cells can be treated with *e.g.*, drugs, compounds and/or small molecules which influence cell-cycle progression so as to arrest cells in G₂ phase. Exemplary molecules of this type include, but are not limited to, compounds which affect microtubule polymerization (*e.g.*, vinblastine, nocodazole, Taxol), compounds that interact with DNA (*e.g.*, *cis*-platinum(II) diamine dichloride, Cisplatin, doxorubicin) and/or compounds that
25 affect DNA synthesis (*e.g.*, thymidine, hydroxyurea, L-mimosine, etoposide, 5-fluorouracil). Additional increases in recombination efficiency are achieved by the use of histone deacetylase (HDAC) inhibitors (*e.g.*, sodium butyrate, trichostatin) which alter chromatin structure to make genomic DNA more accessible to the cellular recombination machinery.

30 Additional methods for cell-cycle arrest include overexpression of proteins (*e.g.*, *cdc28*) which inhibit the activity of the CDK cell-cycle kinases, for example, by

introducing a cDNA encoding the protein into the cell or by introducing into the cell an engineered ZFP which activates expression of the gene encoding the protein. Cell-cycle arrest is also achieved by inhibiting the activity of cyclins and CDKs, for example, using RNAi methods (e.g., U.S. Patent No. 6,506,559) or by introducing into the cell an engineered ZFP which represses expression of one or more genes involved in cell-cycle progression such as, for example, cyclin and/or CDK genes. See, e.g., co-owned U.S. Patent No. 6,534,261 for methods for the synthesis of engineered zinc finger proteins for regulation of gene expression.

Alternatively, in certain cases, targeted cleavage is conducted in the absence of a donor polynucleotide (preferably in S or G₂ phase), and recombination occurs between sister chromatids.

Expression vectors

A nucleic acid encoding one or more ZFPs or ZFP fusion proteins can be cloned into a vector for transformation into prokaryotic or eukaryotic cells for replication and/or expression. Vectors can be prokaryotic vectors, e.g., plasmids, or shuttle vectors, insect vectors, or eukaryotic vectors. A nucleic acid encoding a ZFP can also be cloned into an expression vector, for administration to a plant cell, animal cell, preferably a mammalian cell or a human cell, fungal cell, bacterial cell, or protozoal cell.

To obtain expression of a cloned gene or nucleic acid, sequences encoding a ZFP or ZFP fusion protein are typically subcloned into an expression vector that contains a promoter to direct transcription. Suitable bacterial and eukaryotic promoters are well known in the art and described, e.g., in Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989; 3rd ed., 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, *supra*. Bacterial expression systems for expressing the ZFP are available in, e.g., *E. coli*, *Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983)). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known by those of skill in the art and are also commercially available.

The promoter used to direct expression of a ZFP-encoding nucleic acid depends on the particular application. For example, a strong constitutive promoter is typically used for expression and purification of ZFP. In contrast, when a ZFP is administered *in vivo* for gene regulation, either a constitutive or an inducible promoter is used, depending on the particular use of the ZFP. In addition, a preferred promoter for administration of a ZFP can be a weak promoter, such as HSV TK or a promoter having similar activity.

The promoter typically can also include elements that are responsive to transactivation, *e.g.*, hypoxia response elements, Gal4 response elements, lac repressor response element, and small molecule control systems such as tet-regulated systems and the RU-486 system (see, *e.g.*, Gossen & Bujard, *PNAS* 89:5547 (1992); Oligino *et al.*, *Gene Ther.* 5:491-496 (1998); Wang *et al.*, *Gene Ther.* 4:432-441 (1997); Neering *et al.*, *Blood* 88:1147-1155 (1996); and Rendahl *et al.*, *Nat. Biotechnol.* 16:757-761 (1998)).

In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the nucleic acid in host cells, either prokaryotic or eukaryotic. A typical expression cassette thus contains a promoter operably linked, *e.g.*, to a nucleic acid sequence encoding the ZFP, and signals required, *e.g.*, for efficient polyadenylation of the transcript, transcriptional termination, ribosome binding sites, or translation termination. Additional elements of the cassette may include, *e.g.*, enhancers, and heterologous splicing signals.

The particular expression vector used to transport the genetic information into the cell is selected with regard to the intended use of the ZFP, *e.g.*, expression in plants, animals, bacteria, fungus, protozoa, *etc.* (see expression vectors described below). Standard bacterial expression vectors include plasmids such as pBR322-based plasmids, pSKF, pET23D, and commercially available fusion expression systems such as GST and LacZ. An exemplary fusion protein is the maltose binding protein, "MBP." Such fusion proteins are used for purification of the ZFP. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, for monitoring expression, and for monitoring cellular and subcellular localization, *e.g.*, c-myc or FLAG.

Expression vectors containing regulatory elements from eukaryotic viruses are often used in eukaryotic expression vectors, *e.g.*, SV40 vectors, papilloma virus vectors,

and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 late promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Some expression systems have markers for selection of stably transfected cell lines such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase. High yield expression systems are also suitable, such as using a baculovirus vector in insect cells, with a ZFP encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of recombinant sequences.

Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of protein, which are then purified using standard techniques (*see, e.g., Colley et al., J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification, in Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g., Morrison, J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983).

Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, naked DNA, plasmid vectors, viral vectors, both episomal and integrative, and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see, e.g., Sambrook et al., supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the protein of choice.

Nucleic acids encoding fusion proteins and delivery to cells

Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding engineered ZFPs in cells (e.g., mammalian cells) and target tissues. Such methods can also be used to administer nucleic acids encoding ZFPs to cells *in vitro*. In certain embodiments, nucleic acids encoding ZFPs are administered for *in vivo* or *ex vivo* gene therapy uses. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) (1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994).

Methods of non-viral delivery of nucleic acids encoding engineered ZFPs include electroporation, lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in e.g., US 5,049,386, US 4,946,787; and US 4,897,355) and lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells (*ex vivo* administration) or target tissues (*in vivo* administration).

The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (see, e.g., Crystal, *Science* 270:404-410 (1995); Blaese *et al.*, *Cancer Gene Ther.* 2:291-297 (1995); Behr *et al.*, *Bioconjugate Chem.* 5:382-389 (1994); Remy *et al.*, *Bioconjugate Chem.* 5:647-654 (1994); Gao *et al.*, *Gene Therapy* 2:710-722 (1995); Ahmad *et al.*, *Cancer Res.* 52:4817-

4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

The use of RNA or DNA viral based systems for the delivery of nucleic acids encoding engineered ZFPs take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (*in vivo*) or they can be used to treat cells *in vitro* and the modified cells are administered to patients (*ex vivo*). Conventional viral based systems for the delivery of ZFPs include, but are not limited to, retroviral, lentivirus, adenoviral, adeno-associated, vaccinia and herpes simplex virus vectors for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system depends on the target tissue. Retroviral vectors are comprised of *cis*-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum *cis*-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immunodeficiency virus (SIV), human immunodeficiency virus (HIV), and combinations thereof (*see, e.g.,* Buchscher *et al., J. Virol.* 66:2731-2739 (1992); Johann *et al., J. Virol.* 66:1635-1640 (1992); Sommerfelt *et al., Virol.* 176:58-59 (1990); Wilson *et al., J. Virol.* 63:2374-2378 (1989); Miller *et al., J. Virol.* 65:2220-2224 (1991); PCT/US94/05700).

In applications in which transient expression of a ZFP fusion protein is preferred, adenoviral based systems can be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and high levels of expression have been obtained. This vector can be

produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors are also used to transduce cells with target nucleic acids, e.g., in the *in vitro* production of nucleic acids and peptides, and for *in vivo* and *ex vivo* gene therapy procedures (see, e.g., West *et al.*, *Virology* 160:38-47 (1987); U.S. Patent No. 4,797,368; WO 93/24641; Kotin, *Human Gene Therapy* 5:793-801 (1994); Muzyczka, *J. Clin. Invest.* 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, *et al.*, *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *PNAS* 81:6466-6470 (1984); and Samulski *et al.*, *J. Virol.* 63:03822-3828 (1989).

At least six viral vector approaches are currently available for gene transfer in clinical trials, which utilize approaches that involve complementation of defective vectors by genes inserted into helper cell lines to generate the transducing agent.

pLASN and MFG-S are examples of retroviral vectors that have been used in clinical trials (Dunbar *et al.*, *Blood* 85:3048-305 (1995); Kohn *et al.*, *Nat. Med.* 1:1017-102 (1995); Malech *et al.*, *PNAS* 94:22 12133-12138 (1997)). PA317/pLASN was the first therapeutic vector used in a gene therapy trial. (Blaese *et al.*, *Science* 270:475-480 (1995)). Transduction efficiencies of 50% or greater have been observed for MFG-S packaged vectors. (Ellem *et al.*, *Immunol Immunother.* 44(1):10-20 (1997); Dranoff *et al.*, *Hum. Gene Ther.* 1:111-2 (1997).

Recombinant adeno-associated virus vectors (rAAV) are a promising alternative gene delivery systems based on the defective and nonpathogenic parvovirus adeno-associated type 2 virus. All vectors are derived from a plasmid that retains only the AAV 145 bp inverted terminal repeats flanking the transgene expression cassette. Efficient gene transfer and stable transgene delivery due to integration into the genomes of the transduced cell are key features for this vector system. (Wagner *et al.*, *Lancet* 351:9117 1702-3 (1998), Kearns *et al.*, *Gene Ther.* 9:748-55 (1996)).

Replication-deficient recombinant adenoviral vectors (Ad) can be produced at high titer and readily infect a number of different cell types. Most adenovirus vectors are engineered such that a transgene replaces the Ad E1a, E1b, and/or E3 genes; subsequently the replication defective vector is propagated in human 293 cells that supply

deleted gene function in *trans*. Ad vectors can transduce multiple types of tissues *in vivo*, including nondividing, differentiated cells such as those found in liver, kidney and muscle. Conventional Ad vectors have a large carrying capacity. An example of the use of an Ad vector in a clinical trial involved polynucleotide therapy for antitumor

immunization with intramuscular injection (Stermann *et al.*, *Hum. Gene Ther.* 7:1083-9 (1998)). Additional examples of the use of adenovirus vectors for gene transfer in clinical trials include Rosenecker *et al.*, *Infection* 24:1 5-10 (1996); Stermann *et al.*, *Hum. Gene Ther.* 9:7 1083-1089 (1998); Welsh *et al.*, *Hum. Gene Ther.* 2:205-18 (1995); Alvarez *et al.*, *Hum. Gene Ther.* 5:597-613 (1997); Topf *et al.*, *Gene Ther.* 5:507-513 (1998); Stermann *et al.*, *Hum. Gene Ther.* 7:1083-1089 (1998).

Packaging cells are used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ 2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by a producer cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host (if applicable), other viral sequences being replaced by an expression cassette encoding the protein to be expressed. The missing viral functions are supplied *in trans* by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess inverted terminal repeat (ITR) sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely *rep* and *cap*, but lacking ITR sequences. The cell line is also infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV.

In many gene therapy applications, it is desirable that the gene therapy vector be delivered with a high degree of specificity to a particular tissue type. Accordingly, a viral vector can be modified to have specificity for a given cell type by expressing a ligand as a fusion protein with a viral coat protein on the outer surface of the virus. The ligand is chosen to have affinity for a receptor known to be present on the cell type of interest. For

example, Han *et al.*, *Proc. Natl. Acad. Sci. USA* 92:9747-9751 (1995), reported that Moloney murine leukemia virus can be modified to express human heregulin fused to gp70, and the recombinant virus infects certain human breast cancer cells expressing human epidermal growth factor receptor. This principle can be extended to other virus-target cell pairs, in which the target cell expresses a receptor and the virus expresses a fusion protein comprising a ligand for the cell-surface receptor. For example, filamentous phage can be engineered to display antibody fragments (e.g., Fab or Fv) having specific binding affinity for virtually any chosen cellular receptor. Although the above description applies primarily to viral vectors, the same principles can be applied to nonviral vectors. Such vectors can be engineered to contain specific uptake sequences which favor uptake by specific target cells.

Gene therapy vectors can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (e.g., intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (e.g., lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

Ex vivo cell transfection for diagnostics, research, or for gene therapy (e.g., via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In a preferred embodiment, cells are isolated from the subject organism, transfected with a ZFP nucleic acid (gene or cDNA), and re-infused back into the subject organism (e.g., patient). Various cell types suitable for *ex vivo* transfection are well known to those of skill in the art (see, e.g., Freshney *et al.*, *Culture of Animal Cells, A Manual of Basic Technique* (3rd ed. 1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

In one embodiment, stem cells are used in *ex vivo* procedures for cell transfection and gene therapy. The advantage to using stem cells is that they can be differentiated into other cell types *in vitro*, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Methods for differentiating CD34+ cells *in vitro* into clinically important immune cell types using cytokines such a

GM-CSF, IFN- γ and TNF- α are known (see Inaba *et al.*, *J. Exp. Med.* 176:1693-1702 (1992)).

Stem cells are isolated for transduction and differentiation using known methods. For example, stem cells are isolated from bone marrow cells by panning the bone marrow cells with antibodies which bind unwanted cells, such as CD4+ and CD8+ (T cells), CD45+ (panB cells), GR-1 (granulocytes), and Iad (differentiated antigen presenting cells) (see Inaba *et al.*, *J. Exp. Med.* 176:1693-1702 (1992)).

Vectors (e.g., retroviruses, adenoviruses, liposomes, etc.) containing therapeutic ZFP nucleic acids can also be administered directly to an organism for transduction of cells *in vivo*. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells including, but not limited to, injection, infusion, topical application and electroporation. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions available, as described below (see, e.g., *Remington's Pharmaceutical Sciences*, 17th ed., 1989).

DNA constructs may be introduced into the genome of a desired plant host by a variety of conventional techniques. For reviews of such techniques see, for example, Weissbach & Weissbach *Methods for Plant Molecular Biology* (1988, Academic Press, N.Y.) Section VIII, pp. 421-463; and Grierson & Corey, *Plant Molecular Biology* (1988, 2d Ed.), Blackie, London, Ch. 7-9. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using biolistic methods, such as DNA particle bombardment (see, e.g., Klein *et al* (1987) *Nature* 327:70-73). Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional

Agrobacterium tumefaciens host vector. *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, for example Horsch et al (1984) *Science* 233:496-498, and Fraley et al (1983) *Proc. Nat'l. Acad. Sci. USA* 80:4803. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria using binary T DNA vector (Bevan (1984) *Nuc. Acid Res.* 12:8711-8721) or the co-cultivation procedure (Horsch et al (1985) *Science* 227:1229-1231). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants (Bevan et al (1982) *Ann. Rev. Genet* 16:357-384; Rogers et al (1986) *Methods Enzymol.* 118:627-641). The *Agrobacterium* transformation system may also be used to transform, as well as transfer, DNA to monocotyledonous plants and plant cells. See Hernalsteen et al (1984) *EMBO J* 3:3039-3041; Hooykass-Van Slogteren et al (1984) *Nature* 311:763-764; Grimsley et al (1987) *Nature* 325:1677-179; Boulton et al (1989) *Plant Mol. Biol.* 12:31-40.; and Gould et al (1991) *Plant Physiol.* 95:426-434.

Alternative gene transfer and transformation methods include, but are not limited to, protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (see Paszkowski et al. (1984) *EMBO J* 3:2717-2722, Potrykus et al. (1985) *Molec. Gen. Genet.* 199:169-177; Fromm et al. (1985) *Proc. Nat. Acad. Sci. USA* 82:5824-5828; and Shimamoto (1989) *Nature* 338:274-276) and electroporation of plant tissues (D'Halluin et al. (1992) *Plant Cell* 4:1495-1505). Additional methods for plant cell transformation include microinjection, silicon carbide mediated DNA uptake (Kaepler et al. (1990) *Plant Cell Reporter* 9:415-418), and microprojectile bombardment (see Klein et al. (1988) *Proc. Nat. Acad. Sci. USA* 85:4305-4309; and Gordon-Kamm et al. (1990) *Plant Cell* 2:603-618).

Transformed plant cells which are produced by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described

in Evans, et al., "Protoplasts Isolation and Culture" in *Handbook of Plant Cell Culture*, pp. 124-176, Macmillian Publishing Company, New York, 1983; and Binding, *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, pollens, embryos or parts thereof. Such regeneration techniques are described generally in Klee et al (1987) *Ann. Rev. of Plant Phys.* 38:467-486.

Nucleic acids introduced into a plant cell can be used to confer desired traits on essentially any plant. A wide variety of plants and plant cell systems may be engineered for the desired physiological and agronomic characteristics described herein using the nucleic acid constructs of the present disclosure and the various transformation methods mentioned above. In preferred embodiments, target plants and plant cells for engineering include, but are not limited to, those monocotyledonous and dicotyledonous plants, such as crops including grain crops (e.g., wheat, maize, rice, millet, barley), fruit crops (e.g., tomato, apple, pear, strawberry, orange), forage crops (e.g., alfalfa), root vegetable crops (e.g., carrot, potato, sugar beets, yam), leafy vegetable crops (e.g., lettuce, spinach); flowering plants (e.g., petunia, rose, chrysanthemum), conifers and pine trees (e.g., pine fir, spruce); plants used in phytoremediation (e.g., heavy metal accumulating plants); oil crops (e.g., sunflower, rape seed) and plants used for experimental purposes (e.g., *Arabidopsis*). Thus, the disclosed methods and compositions have use over a broad range of plants, including, but not limited to, species from the genera *Asparagus*, *Avena*, *Brassica*, *Citrus*, *Citrullus*, *Capsicum*, *Cucurbita*, *Daucus*, *Glycine*, *Hordeum*, *Lactuca*, *Lycopersicon*, *Malus*, *Manihot*, *Nicotiana*, *Oryza*, *Persea*, *Pisum*, *Pyrus*, *Prunus*, *Raphanus*, *Secale*, *Solanum*, *Sorghum*, *Triticum*, *Vitis*, *Vigna*, and *Zea*.

One of skill in the art will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

A transformed plant cell, callus, tissue or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection may be performed by growing the engineered plant material on media containing an inhibitory amount of the

antibiotic or herbicide to which the transforming gene construct confers resistance. Further, transformed plants and plant cells may also be identified by screening for the activities of any visible marker genes (e.g., the β -glucuronidase, luciferase, B or C1 genes) that may be present on the recombinant nucleic acid constructs. Such selection and screening methodologies are well known to those skilled in the art.

Physical and biochemical methods also may be used to identify plant or plant cell transformants containing inserted gene constructs. These methods include but are not limited to: 1) Southern analysis or PCR amplification for detecting and determining the structure of the recombinant DNA insert; 2) Northern blot, S1 RNase protection, primer-extension or reverse transcriptase-PCR amplification for detecting and examining RNA transcripts of the gene constructs; 3) enzymatic assays for detecting enzyme or ribozyme activity, where such gene products are encoded by the gene construct; 4) protein gel electrophoresis, Western blot techniques, immunoprecipitation, or enzyme-linked immunoassays, where the gene construct products are proteins. Additional techniques, such as *in situ* hybridization, enzyme staining, and immunostaining, also may be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. The methods for doing all these assays are well known to those skilled in the art.

Effects of gene manipulation using the methods disclosed herein can be observed by, for example, northern blots of the RNA (e.g., mRNA) isolated from the tissues of interest. Typically, if the amount of mRNA has increased, it can be assumed that the corresponding endogenous gene is being expressed at a greater rate than before. Other methods of measuring gene and/or CYP74B activity can be used. Different types of enzymatic assays can be used, depending on the substrate used and the method of detecting the increase or decrease of a reaction product or by-product. In addition, the levels of and/or CYP74B protein expressed can be measured immunochemically, i.e., ELISA, RIA, EIA and other antibody based assays well known to those of skill in the art, such as by electrophoretic detection assays (either with staining or western blotting). The transgene may be selectively expressed in some tissues of the plant or at some developmental stages, or the transgene may be expressed in substantially all plant tissues,

substantially along its entire life cycle. However, any combinatorial expression mode is also applicable.

- 5 The present disclosure also encompasses seeds of the transgenic plants described above wherein the seed has the transgene or gene construct. The present disclosure further encompasses the progeny, clones, cell lines or cells of the transgenic plants described above wherein said progeny, clone, cell line or cell has the transgene or gene construct.

Delivery vehicles

- 10 An important factor in the administration of polypeptide compounds, such as ZFP fusion proteins, is ensuring that the polypeptide has the ability to traverse the plasma membrane of a cell, or the membrane of an intra-cellular compartment such as the nucleus. Cellular membranes are composed of lipid-protein bilayers that are freely permeable to small, nonionic lipophilic compounds and are inherently impermeable to
15 polar compounds, macromolecules, and therapeutic or diagnostic agents. However, proteins and other compounds such as liposomes have been described, which have the ability to translocate polypeptides such as ZFPs across a cell membrane.

- For example, "membrane translocation polypeptides" have amphiphilic or hydrophobic amino acid subsequences that have the ability to act as membrane-
20 translocating carriers. In one embodiment, homeodomain proteins have the ability to translocate across cell membranes. The shortest internalizable peptide of a homeodomain protein, Antennapedia, was found to be the third helix of the protein, from amino acid position 43 to 58 (*see, e.g., Prochiantz, Current Opinion in Neurobiology* 6:629-634 (1996)). Another subsequence, the h (hydrophobic) domain of signal peptides, was found
25 to have similar cell membrane translocation characteristics (*see, e.g., Lin et al., J. Biol. Chem.* 270:1 4255-14258 (1995)).

- Examples of peptide sequences which can be linked to a protein, for facilitating uptake of the protein into cells, include, but are not limited to: an 11 amino acid peptide of the tat protein of HIV; a 20 residue peptide sequence which corresponds to amino
30 acids 84-103 of the p16 protein (*see Fahraeus et al., Current Biology* 6:84 (1996)); the third helix of the 60-amino acid long homeodomain of Antennapedia (Derossi *et al., J.*

Biol. Chem. 269:10444 (1994)); the h region of a signal peptide such as the Kaposi fibroblast growth factor (K-FGF) h region (Lin *et al.*, *supra*); or the VP22 translocation domain from HSV (Elliot & O'Hare, *Cell* 88:223-233 (1997)). Other suitable chemical moieties that provide enhanced cellular uptake may also be chemically linked to ZFPs.

- 5 Membrane translocation domains (*i.e.*, internalization domains) can also be selected from libraries of randomized peptide sequences. *See*, for example, Yeh *et al.* (2003) *Molecular Therapy* 7(5):S461, Abstract #1191.

- Toxin molecules also have the ability to transport polypeptides across cell membranes. Often, such molecules (called "binary toxins") are composed of at least two parts: a translocation/binding domain or polypeptide and a separate toxin domain or polypeptide. Typically, the translocation domain or polypeptide binds to a cellular receptor, and then the toxin is transported into the cell. Several bacterial toxins, including *Clostridium perfringens* iota toxin, diphtheria toxin (DT), *Pseudomonas* exotoxin A (PE), pertussis toxin (PT), *Bacillus anthracis* toxin, and pertussis adenylate cyclase (CYA), have been used to deliver peptides to the cell cytosol as internal or amino-terminal fusions (Arora *et al.*, *J. Biol. Chem.*, 268:3334-3341 (1993); Perelle *et al.*, *Infect. Immun.*, 61:5147-5156 (1993); Stenmark *et al.*, *J. Cell Biol.* 113:1025-1032 (1991); Donnelly *et al.*, *PNAS* 90:3530-3534 (1993); Carbonetti *et al.*, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 95:295 (1995); Sebo *et al.*, *Infect. Immun.* 63:3851-3857 (1995);
20 Klimpel *et al.*, *PNAS U.S.A.* 89:10277-10281 (1992); and Novak *et al.*, *J. Biol. Chem.* 267:17186-17193 (1992)).

- Such peptide sequences can be used to translocate ZFPs across a cell membrane. ZFPs can be conveniently fused to or derivatized with such sequences. Typically, the translocation sequence is provided as part of a fusion protein. Optionally, a linker can be used to link the ZFP and the translocation sequence. Any suitable linker can be used, e.g., a peptide linker.

- The ZFP can also be introduced into an animal cell, preferably a mammalian cell, via a liposomes and liposome derivatives such as immunoliposomes. The term "liposome" refers to vesicles comprised of one or more concentrically ordered lipid bilayers, which encapsulate an aqueous phase. The aqueous phase typically contains the compound to be delivered to the cell, *i.e.*, a ZFP.

The liposome fuses with the plasma membrane, thereby releasing the drug into the cytosol. Alternatively, the liposome is phagocytosed or taken up by the cell in a transport vesicle. Once in the endosome or phagosome, the liposome either degrades or fuses with the membrane of the transport vesicle and releases its contents.

5 In current methods of drug delivery via liposomes, the liposome ultimately becomes permeable and releases the encapsulated compound (in this case, a ZFP) at the target tissue or cell. For systemic or tissue specific delivery, this can be accomplished, for example, in a passive manner wherein the liposome bilayer degrades over time through the action of various agents in the body. Alternatively, active drug release
10 involves using an agent to induce a permeability change in the liposome vesicle. Liposome membranes can be constructed so that they become destabilized when the environment becomes acidic near the liposome membrane (*see, e.g., PNAS* 84:7851 (1987); *Biochemistry* 28:908 (1989)). When liposomes are endocytosed by a target cell, for example, they become destabilized and release their contents. This destabilization is
15 termed fusogenesis. Dioleoylphosphatidylethanolamine (DOPE) is the basis of many "fusogenic" systems.

Such liposomes typically comprise a ZFP and a lipid component, e.g., a neutral and/or cationic lipid, optionally including a receptor-recognition molecule such as an antibody that binds to a predetermined cell surface receptor or ligand (*e.g., an antigen*). A
20 variety of methods are available for preparing liposomes as described in, *e.g., Szoka et al., Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,946,787, PCT Publication No. WO 91/17424, Deamer & Bangham, *Biochim. Biophys. Acta* 443:629-634 (1976); Fraley, *et al., PNAS* 76:3348-3352 (1979); Hope *et al., Biochim. Biophys. Acta* 812:55-65 (1985);
25 Mayer *et al., Biochim. Biophys. Acta* 858:161-168 (1986); Williams *et al., PNAS* 85:242-246 (1988); *Liposomes* (Ostro (ed.), 1983, Chapter 1); Hope *et al., Chem. Phys. Lip.* 40:89 (1986); Gregoriadis, *Liposome Technology* (1984) and Lasic, *Liposomes: from Physics to Applications* (1993)). Suitable methods include, for example, sonication,
30 extrusion, high pressure/homogenization, microfluidization, detergent dialysis, calcium-

induced fusion of small liposome vesicles and ether-fusion methods, all of which are known to those of skill in the art.

In certain embodiments, it is desirable to target liposomes using targeting moieties that are specific to a particular cell type, tissue, and the like. Targeting of liposomes using a variety of targeting moieties (e.g., ligands, receptors, and monoclonal antibodies) has been described. See, e.g., U.S. Patent Nos. 4,957,773 and 4,603,044.

Examples of targeting moieties include monoclonal antibodies specific to antigens associated with neoplasms, such as prostate cancer specific antigen and MAGE. Tumors can also be diagnosed by detecting gene products resulting from the activation or over-expression of oncogenes, such as ras or c-erbB2. In addition, many tumors express antigens normally expressed by fetal tissue, such as the alphafetoprotein (AFP) and carcinoembryonic antigen (CEA). Sites of viral infection can be diagnosed using various viral antigens such as hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, human immunodeficiency type-1 virus (HIV1) and papilloma virus antigens. Inflammation can be detected using molecules specifically recognized by surface molecules which are expressed at sites of inflammation such as integrins (e.g., VCAM-1), selectin receptors (e.g., ELAM-1) and the like.

Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes of lipid components, e.g., phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid derivatized bleomycin. Antibody targeted liposomes can be constructed using, for instance, liposomes which incorporate protein A (see Renneisen *et al.*, *J. Biol. Chem.*, 265:16337-16342 (1990) and Leonetti *et al.*, *PNAS* 87:2448-2451 (1990).

Dosages

For therapeutic applications, the dose administered to a patient, in the context of the present disclosure, should be sufficient to effect a beneficial therapeutic response in the patient over time. In addition, particular dosage regimens can be useful for determining phenotypic changes in an experimental setting, e.g., in functional genomics studies, and in cell or animal models. The dose will be determined by the efficacy and K_d

of the particular ZFP employed, the nuclear volume of the target cell, and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular compound or vector in a particular patient.

The maximum therapeutically effective dosage of ZFP for approximately 99% binding to target sites is calculated to be in the range of less than about 1.5×10^5 to 1.5×10^6 copies of the specific ZFP molecule per cell. The number of ZFPs per cell for this level of binding is calculated as follows, using the volume of a HeLa cell nucleus

(approximately $1000 \mu\text{m}^3$ or 10^{-12} L; *Cell Biology*, (Altman & Katz, eds. (1976)). As the HeLa nucleus is relatively large, this dosage number is recalculated as needed using the volume of the target cell nucleus. This calculation also does not take into account competition for ZFP binding by other sites. This calculation also assumes that essentially all of the ZFP is localized to the nucleus. A value of $100 \times K_d$ is used to calculate approximately 99% binding of to the target site, and a value of $10 \times K_d$ is used to calculate approximately 90% binding of to the target site. For this example, $K_d = 25 \text{ nM}$

$\text{ZFP} + \text{target site} \leftrightarrow \text{complex}$

i.e., $\text{DNA} + \text{protein} \leftrightarrow \text{DNA:protein complex}$

$K_d = \frac{[\text{DNA}] [\text{protein}]}{[\text{DNA:protein complex}]}$

When 50% of ZFP is bound, $K_d = [\text{protein}]$

So when $[\text{protein}] = 25 \text{ nM}$ and the nucleus volume is 10^{-12} L

$[\text{protein}] = (25 \times 10^{-9} \text{ moles/L}) (10^{-12} \text{ L/nucleus}) (6 \times 10^{23})$

molecules/mole)

= 15,000 molecules/nucleus for 50% binding

When 99% target is bound; $100 \times K_d = [\text{protein}]$

$100 \times K_d = [\text{protein}] = 2.5 \mu\text{M}$

$(2.5 \times 10^{-6} \text{ moles/L}) (10^{-12} \text{ L/nucleus}) (6 \times 10^{23} \text{ molecules/mole})$

= about 1,500,000 molecules per nucleus for 99% binding of target

site.

The appropriate dose of an expression vector encoding a ZFP can also be calculated by taking into account the average rate of ZFP expression from the promoter and the average rate of ZFP degradation in the cell. In certain embodiments, a weak promoter such as a wild-type or mutant HSV TK promoter is used, as described above.

- 5 The dose of ZFP in micrograms is calculated by taking into account the molecular weight of the particular ZFP being employed.

- In determining the effective amount of the ZFP to be administered in the treatment or prophylaxis of disease, the physician evaluates circulating plasma levels of the ZFP or nucleic acid encoding the ZFP, potential ZFP toxicities, progression of the
10 disease, and the production of anti-ZFP antibodies. Administration can be accomplished via single or divided doses.

Pharmaceutical compositions and administration

- ZFPs and expression vectors encoding ZFPs can be administered directly to the
15 patient for targeted cleavage and/or recombination, and for therapeutic or prophylactic applications, for example, cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, and the like. Examples of microorganisms that can be inhibited by ZFP gene therapy
20 include pathogenic bacteria, e.g., chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptococci, pneumococci, meningococci and conococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lyme disease bacteria; infectious fungus, e.g., *Aspergillus*, *Candida* species; protozoa such as sporozoa (e.g., *Plasmodia*),
25 rhizopods (e.g., *Entamoeba*) and flagellates (*Trypanosoma*, *Leishmania*, *Trichomonas*, *Giardia*, etc.); viral diseases, e.g., hepatitis (A, B, or C), herpes virus (e.g., VZV, HSV-1, HSV-6, HSV-II, CMV, and EBV), HIV, Ebola, adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, coronavirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus,
30 dengue virus, papillomavirus, poliovirus, rabies virus, and arboviral encephalitis virus, etc.

Administration of therapeutically effective amounts is by any of the routes normally used for introducing ZFP into ultimate contact with the tissue to be treated. The ZFPs are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable methods of administering such modulators are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions that are available (*see, e.g., Remington's Pharmaceutical Sciences*, 17th ed. 1985)).

The ZFPs, alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Formulations suitable for parenteral administration, such as, for example, by intravenous, intramuscular, intradermal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The disclosed compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

Applications

The disclosed methods and compositions for targeted cleavage can be used to induce mutations in a genomic sequence, *e.g.*, by cleaving at two sites and deleting

sequences in between, by cleavage at a single site followed by non-homologous end joining, and/or by cleaving at a site so as to remove one or two or a few nucleotides. Targeted cleavage can also be used to create gene knock-outs (*e.g.*, for functional genomics or target validation) and to facilitate targeted insertion of a sequence into a genome (*i.e.*, gene knock-in); *e.g.*, for purposes of cell engineering or protein overexpression.

Targeted cleavage of infecting or integrated viral genomes can be used to treat viral infections in a host. Additionally, targeted cleavage of genes encoding receptors for viruses can be used to block expression of such receptors, thereby preventing viral infection and/or viral spread in a host organism. Non-limiting examples of viruses or viral receptors that may be targeted include herpes simplex virus (HSV), such as HSV-1 and HSV-2, varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV), HHV6 and HHV7. The hepatitis family of viruses includes hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV). Other viruses or their receptors may be targeted, including, but not limited to, Picornaviridae (*e.g.*, polioviruses, etc.); Caliciviridae; Togaviridae (*e.g.*, rubella virus, dengue virus, *etc.*); Flaviviridae; Coronaviridae; Reoviridae; Birnaviridae; Rhabdoviridae (*e.g.*, rabies virus, etc.); Filoviridae; Paramyxoviridae (*e.g.*, mumps virus, measles virus, respiratory syncytial virus, *etc.*); Orthomyxoviridae (*e.g.*, influenza virus types A, B and C, *etc.*); Bunyaviridae; Arenaviridae; Retroviridae (*e.g.*, HTLV-I; HTLV-II; HIV-1 (also known as HTLV-III, LAV, ARV, hTLR, *etc.*)); simian immunodeficiency virus (SIV), human papillomavirus (HPV), influenza virus and the tick-borne encephalitis viruses. See, *e.g.* Virology, 3rd Edition (W. K. Joklik ed. 1988); Fundamental Virology, 2nd Edition (B. N. Fields and D. M. Knipe, eds. 1991), for a description of these and other viruses.

The disclosed methods for targeted recombination can be used to replace any genomic sequence with a homologous, non-identical sequence. For example, a mutant genomic sequence can be replaced by its wild-type counterpart, thereby providing methods for treatment of *e.g.*, genetic disease, inherited disorders, cancer, and autoimmune disease. :

Exemplary genetic diseases include, but are not limited to, achondroplasia, achromatopsia, acid maltase deficiency, adrenoleukodystrophy, aicardi syndrome, alpha-1 antitrypsin deficiency, androgen insensitivity syndrome, apert syndrome, arrhythmogenic right ventricular, dysplasia, ataxia telangiectasia, Barth syndrome, blue rubber bleb nevus syndrome, Canavan disease, cri du chat syndrome, cystic fibrosis, dercun's disease, ectodermal dysplasia, Fanconi anemia, fibrodysplasia ossificans progressive, fragile X syndrome, galactosemia, Gaucher disease, hemochromatosis, hemophilia, Huntington's disease, Hurler Syndrome, hypophosphatase, Klinefelter syndrome, Krabbe Disease, Langer-Giedion Syndrome, leukodystrophy, long QT syndrome, Marfan syndrome, Moebius syndrome, mucopolysaccharidosis (MPS), nail patella syndrome, nephrogenic diabetes insipidus, neurofibromatosis, Neimann-Pick disease, osteogenesis imperfecta, porphyria, Prader-Willi syndrome, progeria, Proteus syndrome, retinoblastoma, Rett syndrome, Rubinstein-Taybi syndrome, Sanfilippo syndrome, severe combined immunodeficiency (SCID), Shwachman syndrome, sickle cell disease, Smith-Magenis syndrome, Stickler syndrome, Tay-Sachs, Thrombocytopenia Absent Radius (TAR) syndrome, Treacher Collins syndrome, trisomy, tuberous sclerosis, Turner's syndrome, urea cycle disorder, von Hippel-Landau disease, Waardenburg syndrome, Williams syndrome, and Wilson's disease.

In many of these cases, a region of interest comprises a mutation, and the donor polynucleotide comprises the corresponding wild-type sequence. Similarly, a wild-type genomic sequence can be replaced by a mutant sequence, if such is desirable. For example, overexpression of an oncogene can be reversed either by mutating the gene or by replacing its control sequences with sequences that support a lower, non-pathologic level of expression. Indeed, any pathology dependent upon a particular genomic sequence, in any fashion, can be corrected or alleviated using the methods and compositions disclosed herein.

Targeted cleavage and targeted recombination can also be used to alter non-coding sequences (e.g., regulatory sequences such as promoters, enhancers, initiators, terminators, splice sites) to alter the levels of expression of a gene product. Such methods can be used, for example, for therapeutic purposes, functional genomics and/or target validation studies.

The compositions and methods described herein also allow for novel approaches and systems to address immune reactions of a host to allogeneic grafts. In particular, a major problem faced when allogeneic stem cells (or any type of allogeneic cell) are grafted into a host recipient is the high risk of rejection by the host's immune system, primarily mediated through recognition of the Major Histocompatibility Complex (MHC) on the surface of the engrafted cells. The MHC comprises the HLA class I protein(s) that function as heterodimers that are comprised of a common β subunit and variable α subunits. It has been demonstrated that tissue grafts derived from stem cells that are devoid of HLA escape the host's immune response. See, e.g., Coffman et al. *J Immunol* **151**, 425-35. (1993); Markmann et al. *Transplantation* **54**, 1085-9. (1992); Koller et al. *Science* **248**, 1227-30. (1990). Using the compositions and methods described herein, genes encoding HLA proteins involved in graft rejection can be cleaved, mutagenized or altered by recombination, in either their coding or regulatory sequences, so that their expression is blocked or they express a non-functional product. For example, by inactivating the gene encoding the common β subunit gene ($\beta 2$ microglobulin) using ZFP fusion proteins as described herein, HLA class I can be removed from the cells to rapidly and reliably generate HLA class I null stem cells from any donor, thereby reducing the need for closely matched donor/recipient MHC haplotypes during stem cell grafting.

Inactivation of any gene (e.g., the $\beta 2$ microglobulin gene) can be achieved, for example, by a single cleavage event, by cleavage followed by non-homologous end joining, by cleavage at two sites followed by joining so as to delete the sequence between the two cleavage sites, by targeted recombination of a missense or nonsense codon into the coding region, or by targeted recombination of an irrelevant sequence (i.e., a "stuffer" sequence) into the gene or its regulatory region, so as to disrupt the gene or regulatory region.

Targeted modification of chromatin structure, as disclosed in co-owned WO 01/83793, can be used to facilitate the binding of fusion proteins to cellular chromatin.

In additional embodiments, one or more fusions between a zinc finger binding domain and a recombinase (or functional fragment thereof) can be used, in addition to or instead of the zinc finger-cleavage domain fusions disclosed herein, to facilitate targeted

recombination. See, for example, co-owned US patent No. 6,534,261 and Akopian *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100**:8688-8691.

In additional embodiments, the disclosed methods and compositions are used to provide fusions of ZFP binding domains with transcriptional activation or repression domains that require dimerization (either homodimerization or heterodimerization) for their activity. In these cases, a fusion polypeptide comprises a zinc finger binding domain and a functional domain monomer (*e.g.*, a monomer from a dimeric transcriptional activation or repression domain). Binding of two such fusion polypeptides to properly situated target sites allows dimerization so as to reconstitute a functional transcription activation or repression domain.

EXAMPLES

Example 1: Editing of a Chromosomal hSMC1L1 Gene by Targeted Recombination

The hSMC1L1 gene is the human orthologue of the budding yeast gene *structural maintenance of chromosomes 1*. A region of this gene encoding an amino-terminal portion of the protein which includes the Walker ATPase domain was mutagenized by targeted cleavage and recombination. Cleavage was targeted to the region of the methionine initiation codon (nucleotides 24-26, Figure 1), by designing chimeric nucleases, comprising a zinc finger DNA-binding domain and a *FokI* cleavage half-domain, which bind in the vicinity of the codon. Thus, two zinc finger binding domains were designed, one of which recognizes nucleotides 23-34 (primary contacts along the top strand as shown in Figure 1), and the other of which recognizes nucleotides 5-16 (primary contacts along the bottom strand). Zinc finger proteins were designed as described in co-owned US Patents 6,453,242 and 6,534,261. See Table 2 for the amino acid sequences of the recognition regions of the zinc finger proteins.

Sequences encoding each of these two ZFP binding domains were fused to sequences encoding a *FokI* cleavage half-domain (amino acids 384-579 of the native *FokI* sequence; Kita *et al.* (1989) *J. Biol. Chem.* **264**:5751-5756), such that the encoded protein contained *FokI* sequences at the carboxy terminus and ZFP sequences at the

amino terminus. Each of these fusion sequences was then cloned in a modified mammalian expression vector pcDNA3 (Figure 2).

Table 2: Zinc Finger Designs for the hSMC1L1 Gene

Target sequence	F1	F2	F3	F4
CATGGGGTTCCT (SEQ ID NO: 1)	RSHDLIE (SEQ ID NO: 2)	TSSSLSR (SEQ ID NO: 3)	RSDHLST (SEQ ID NO: 4)	TNSNRIT (SEQ ID NO: 5)
GCGGCGCCGGCG (SEQ ID NO: 6)	RSDDLRS (SEQ ID NO: 7)	RSDDRKT (SEQ ID NO: 8)	RSEDLIR (SEQ ID NO: 9)	RSDTLRS (SEQ ID NO: 10)

Note: The zinc finger amino acid sequences shown above (in one-letter code) represent residues -1 through +6, with respect to the start of the alpha-helical portion of each zinc finger. Finger F1 is closest to the amino terminus of the protein, and Finger F4 is closest to the carboxy terminus.

A donor DNA molecule was obtained as follows. First, a 700 base pair fragment of human genomic DNA representing nucleotides 52415936-52416635 of the "-" strand of the X chromosome (UCSC human genome release July, 2003), which includes the first exon of the human hSMC1L1 gene, was amplified, using genomic DNA from HEK293 cells as template. Sequences of primers used for amplification are shown in Table 3 ("Initial amp 1" and "Initial amp 2"). The PCR product was then altered, using standard overlap extension PCR methodology (see, *e.g.*, Ho, *et al.* (1989) *Gene* 77:51-59), resulting in replacement of the sequence ATGGGG (nucleotides 24-29 in Figure 1) to ATAAGAAGC. This change resulted in conversion of the ATG codon (methionine) to an ATA codon (isoleucine) and replacement of GGG (nucleotides 27-29 in Figure 1) by the sequence AGAAGC, allowing discrimination between donor-derived sequences and endogenous chromosomal sequences following recombination. A schematic diagram of the hSMC1 gene, including sequences of the chromosomal DNA in the region of the initiation codon, and sequences in the donor DNA that differ from the chromosomal sequence, is given in Figure 3. The resulting 700 base pair donor fragment was cloned into pCR4BluntTopo, which does not contain any sequences homologous to the human genome. See Figure 4.

For targeted mutation of the chromosomal hSMC1L1 gene, the two plasmids encoding ZFP-*FokI* fusions and the donor plasmid were introduced into 1×10^6 HEK293 cells by transfection using Lipofectamine 2000® (Invitrogen). Controls included cells

transfected only with the two plasmids encoding the ZFP-*FokI* fusions, cells transfected only with the donor plasmid and cells transfected with a control plasmid (pEGFP-N1, Clontech). Cells were cultured in 5% CO₂ at 37°C. At 48 hours after transfection, genomic DNA was isolated from the cells, and 200 ng was used as template for PCR amplification, using one primer complementary to a region of the gene outside of its region of homology with the donor sequences (nucleotides 52416677-52416701 on the "-" STRAND of the X chromosome; UCSC July 2003), and a second primer complementary to a region of the donor molecule into which distinguishing mutations were introduced. Using these two primers, an amplification product of 400 base pairs will be obtained from genomic DNA if a targeted recombination event has occurred. The sequences of these primers are given in Table 3 (labeled "chromosome-specific" and "donor-specific," respectively). Conditions for amplification were: 94°C, 2 min, followed by 40 cycles of 94°C, 30 sec, 60°C, 1 min, 72°C, 1 min; and a final step of 72°C, 7min.

The results of this analysis (Figure 5) indicate that a 400 base pair amplification product (labeled "Chimeric DNA" in the Figure) was obtained only with DNA extracted from cells which had been transfected with the donor plasmid and both ZFP-*FokI* plasmids.

Table 3: Amplification Primers for the hSMC1L1 Gene

Initial amp 1	AGCAACAACCTCCTCCGGGGATC (SEQ ID NO: 11)
Initial amp 2	TTCCAGACGCGACTCTTTGGC (SEQ ID NO: 12)
Chromosome-specific	CTCAGCAAGCGTGAGCTCAGGTCTC (SEQ ID NO: 13)
Donor-specific	CAATCAGTTTCAGGAAGCTTCTT (SEQ ID NO: 14)
Outside 1	CTCAGCAAGCGTGAGCTCAGGTCTC (SEQ ID NO: 15)
Outside 2	GGGGTCAAGTAAGGCTGGAAGC (SEQ ID NO: 16)

To confirm this result, two additional experiments were conducted. First, the amplification product was cloned into pCR4Blunt-Topo (Invitrogen) and its nucleotide sequence was determined. As shown in Figure 6 (SEQ ID NO: **), the amplified sequence obtained from chromosomal DNA of cells transfected with the two ZFP-*FokI*-encoding plasmids and the donor plasmid contains the AAGAAGC sequence that is

unique to the donor (nucleotides 395-401 of the sequence presented in Figure 6) covalently linked to chromosomal sequences not present in the donor molecule (nucleotides 32-97 of Figure 6), indicating that donor sequences have been recombined into the chromosome. In particular, the G→A mutation converting the initiation codon to an isoleucine codon is observed at position 395 in the sequence.

In a second experiment, chromosomal DNA from cells transfected only with donor plasmid, cells transfected with both ZFP-*FokI* fusion plasmids, cells transfected with the donor plasmid and both ZFP-*FokI* fusion plasmids or cells transfected with the EGFP control plasmid was used as template for amplification, using primers complementary to sequences outside of the 700-nucleotide region of homology between donor and chromosomal sequences (identified as “Outside 1” and “Outside 2” in Table 3). The resulting amplification product was purified and used as template for a second amplification reaction using the donor-specific and chromosome-specific primers described above (Table 3). This amplification yielded a 400 nucleotide product only from cells transfected with the donor construct and both ZFP-*FokI* fusion constructs, a result consistent with the replacement of genomic sequences by targeted recombination in these cells.

Example 2: Editing of a Chromosomal IL2R γ Gene by Targeted

Recombination

The IL2R γ gene encodes a protein, known as the “common gamma chain,” that functions as a subunit of several interleukin receptors. Mutations in this gene, including those surrounding the 5' end of the third exon (e.g. the tyrosine 91 codon), can cause X-linked severe combined immunodeficiency (SCID). See, for example, Puck et al. (1997) Blood 89:1968-1977. A mutation in the tyrosine 91 codon (nucleotides 23-25 of Figure 7; SEQ ID NO: ***), was introduced into the IL2R γ gene by targeted cleavage and recombination. Cleavage was targeted to this region by designing two pairs of zinc finger proteins. The first pair (first two rows of Table 4) comprises a zinc finger protein designed to bind to nucleotides 29-40 (primary contacts along the top strand as shown in Figure 7) and a zinc finger protein designed to bind to nucleotides 8-20 (primary contacts along the bottom strand). The second pair (third and fourth rows of Table 4) comprises

two zinc finger proteins, the first of which recognizes nucleotides 23-34 (primary contacts along the top strand as shown in Figure 7) and the second of which recognizes nucleotides 8-16 (primary contacts along the bottom strand). Zinc finger proteins were designed as described in co-owned US Patents 6,453,242 and 6,534,261. See Table 4 for the amino acid sequences of the recognition regions of the zinc finger proteins.

Sequences encoding the ZFP binding domains were fused to sequences encoding a *FokI* cleavage half-domain (amino acids 384-579 of the native *FokI* sequence, Kita *et al.*, *supra*), such that the encoded protein contained *FokI* sequences at the carboxy terminus and ZFP sequences at the amino terminus. Each of these fusion sequences was then cloned in a modified mammalian expression vector pcDNA3. See Figure 8 for a schematic diagram of the constructs.

Table 4: Zinc Finger Designs for the IL2R γ Gene

Target sequence	F1	F2	F3	F4
AACTCGGATAAT (SEQ ID NO:)	DRSTLIE (SEQ ID NO:)	SSSNLSR (SEQ ID NO:)	RSDDLK (SEQ ID NO:)	DNSNRIK (SEQ ID NO:)
TAGAGGaGAAAG G (SEQ ID NO:)	RSDNLSN (SEQ ID NO:)	TSSSRIN (SEQ ID NO:)	RSDHLSQ (SEQ ID NO:)	RNADRKT (SEQ ID NO:)
TACAAGAACTCG (SEQ ID NO:)	RSDDLK (SEQ ID NO:)	DNSNRIK (SEQ ID NO:)	RSDALSV (SEQ ID NO:)	DNANRTK (SEQ ID NO:)
GGAGAAAGG (SEQ ID NO:)	RSDHLTQ (SEQ ID NO:)	QSGNLAR (SEQ ID NO:)	RSDHLSR (SEQ ID NO:)	

Note: The zinc finger amino acid sequences shown above (in one-letter code) represent residues -1 through +6, with respect to the start of the alpha-helical portion of each zinc finger. Finger F1 is closest to the amino terminus of the protein.

A donor DNA molecule was obtained as follows. First, a 700 base pair fragment of human DNA corresponding to positions 69196910-69197609 on the "-" strand of the X chromosome (UCSC, July 2003), which includes exon 3 of the of the IL2R γ gene, was amplified, using genomic DNA from K562 cells as template. See Figure 9. Sequences of primers used for amplification are shown in Table 5 (labeled initial amp 1 and initial amp 2). The PCR product was then altered via standard overlap extension PCR methodology (Ho, *et al.*, *supra*) to replace the sequence TACAAGAACTCGGATAAT (SEQ ID NO: **) with the sequence TAAAGAATTCCGACAAC (SEQ ID NO: **).

This replacement results in the introduction of a point mutation at nucleotide 25 (Figure 7), converting the tyrosine 91 codon TAC to a TAA termination codon and enables discrimination between donor-derived and endogenous chromosomal sequences following recombination, because of differences in the sequences downstream of codon 91. The resulting 700 base pair fragment was cloned into pCR4BluntTopo which does not contain any sequences homologous to the human genome. See Figure 10.

For targeted mutation of the chromosomal IL2R γ gene, the donor plasmid, along with two plasmids each encoding one of a pair of ZFP-*FokI* fusions, were introduced into 2×10^6 K652 cells using mixed lipofection/electroporation (Amaxa). Each of the ZFP/*FokI* pairs (see Table 4) was tested in separate experiments. Controls included cells transfected only with two plasmids encoding ZFP-*FokI* fusions, and cells transfected only with the donor plasmid. Cells were cultured in 5% CO₂ at 37°C. At 48 hours after transfection, genomic DNA was isolated from the cells, and 200 ng was used as template for PCR amplification, using one primer complementary to a region of the gene outside of its region of homology with the donor sequences (nucleotides 69196839-69196863 on the "+" strand of the X chromosome; UCSC, July 2003), and a second primer complementary to a region of the donor molecule into which distinguishing mutations were introduced (see above) and whose sequence therefore diverges from that of chromosomal DNA. See Table 5 for primer sequences, labeled "chromosome-specific" and "donor-specific," respectively. Using these two primers, an amplification product of 500 bp is obtained from genomic DNA in which a targeted recombination event has occurred. Conditions for amplification were: 94°C, 2 min, followed by 35 cycles of 94°C, 30 sec, 62°C, 1 min, 72°C, 45 sec; and a final step of 72°C, 7min.

The results of this analysis (Figure 11) indicate that an amplification product of the expected size (500 base pairs) is obtained with DNA extracted from cells which had been transfected with the donor plasmid and either of the pairs of ZFP-*FokI*-encoding plasmids. DNA from cells transfected with plasmids encoding a pair of ZFPs only (no donor plasmid) did not result in generation of the 500 bp product, nor did DNA from cells transfected only with the donor plasmid..

Table 5: Amplification Primers for the IL2R γ Gene

Initial amp 1	GTGCGAGTACATGAATTGCACTTGG (SEQ ID NO: **)
Initial amp 2	TTAGGTTCTCTGGAGCCCAGGG (SEQ ID NO: **)
Chromosome-specific	CTCCAAACAGTGTTCAAGAATCTG (SEQ ID NO: **)
Donor-specific	TCCTCTAGGTAAAGAATCCGACAAC (SEQ ID NO: **)

To confirm this result, the amplification product obtained from the experiment using the second pair of ZFP/*FokI* fusions was cloned into pCR4Blunt-Topo (Invitrogen) and its nucleotide sequence was determined. As shown in Figure 12 (SEQ ID NO: **), the sequence consists of a fusion between chromosomal sequences and sequences from the donor plasmid. In particular, the G to A mutation converting tyrosine 91 to a stop codon is observed at position 43 in the sequence. Positions 43-58 contain nucleotides unique to the donor; nucleotides 32-42 and 59-459 are sequences common to the donor and the chromosome, and nucleotides 460-552 are unique to the chromosome. The presence of donor-unique sequences covalently linked to sequences present in the chromosome but not in the donor indicates that DNA from the donor plasmid was introduced into the chromosome by homologous recombination.

Example 3: Editing of a Chromosomal β -globin Gene by Targeted Recombination

The human beta globin gene is one of two gene products responsible for the structure and function of hemoglobin in adult human erythrocytes. Mutations in the beta-globin gene can result in sickle cell anemia. Two zinc finger proteins were designed to bind within this sequence, near the location of a nucleotide which, when mutated, causes sickle cell anemia. Figure 13 shows the nucleotide sequence of a portion of the human beta-globin gene, and the target sites for the two zinc finger proteins are underlined in the sequence presented in Figure 13. Amino acid sequences of the recognition regions of the two zinc finger proteins are shown in Table 6. Sequences encoding each of these two ZFP binding domains were fused to sequences encoding a *FokI* cleavage half-domain, as described above, to create engineered ZFP-nucleases that targeted the endogenous beta globin gene. Each of these fusion sequences was then cloned in the mammalian expression vector pcDNA3.1 (Figure 14).

Table 6: Zinc Finger Designs for the beta-globin Gene

Target sequence	F1	F2	F3	F4
GGGCAGTAACGG (SEQ ID NO: **)	RSDHLS (SEQ ID NO: **)	QSANRTK (SEQ ID NO: **)	RSDNLSA (SEQ ID NO: **)	RSQNRTR (SEQ ID NO: **)
AAGGTGAACGTG (SEQ ID NO: **)	RSDSLSR (SEQ ID NO: **)	DSSNRKT (SEQ ID NO: **)	RSDSLSA (SEQ ID NO: **)	RNDNRKT (SEQ ID NO: **)

Note: The zinc finger amino acid sequences shown above (in one-letter code) represent residues -1 through +6, with respect to the start of the alpha-helical portion of each zinc finger. Finger F1 is closest to the amino terminus of the protein, and Finger F4 is closest to the carboxy terminus.

A donor DNA molecule was obtained as follows. First, a 700 base pair fragment of human genomic DNA corresponding to nucleotides 5212134 - 5212833 on the "-" strand of Chromosome 11 (BLAT, UCSC Human Genome site) was amplified by PCR, using genomic DNA from K562 cells as template. Sequences of primers used for amplification are shown in Table 7 (labeled initial amp 1 and initial amp 2). The resulting amplified fragment contains sequences corresponding to the promoter, the first two exons and the first intron of the human beta globin gene. See Figure 15 for a schematic illustrating the locations of exons 1 and 2, the first intron, and the primer binding sites in the beta globin sequence. The cloned product was then further modified by PCR to introduce a set of sequence changes between nucleotides 305-336 (as shown in Figure 13), which replaced the sequence

CCGTTACTGCCCTGTGGGCAAGGTGAACGTG (SEQ ID NO: **) with gCGTTAGTGCCCGAATTCGAtcGTcAACcAc (SEQ ID NO: **) (changes in bold).

Certain of these changes (shown in lowercase) were specifically engineered to prevent the ZFP/*FokI* fusion proteins from binding to and cleaving the donor sequence, once integrated into the chromosome. In addition, all of the sequence changes enable discrimination between donor and endogenous chromosomal sequences following recombination. The resulting 700 base pair fragment was cloned into pCR4-TOPO, which does not contain any sequences homologous to the human genome (Figure 16).

For targeted mutation of the chromosomal beta globin gene, the two plasmids encoding ZFP-*FokI* fusions and the donor plasmid (pCR4-TOPO-HBBdonor) were introduced into 1 X 10⁶ K562 cells by transfection using Nucleofector™ Solution

(Amaya Biosystems). Controls included cells transfected only with 100 ng (low) or 200 ng (high) of the two plasmids encoding the ZFP-*FokI* fusions, cells transfected only with 200 ng (low) or 600 ng (high) of the donor plasmid, cells transfected with a GFP-encoding plasmid, and mock transfected cells. Cells were cultured in RPMI Medium 1640 (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 2 mM L-glutamine. Cells were maintained at 37°C in an atmosphere of 5% CO₂. At 72 hours after transfection, genomic DNA was isolated from the cells, and 200 ng was used as template for PCR amplification, using one primer complementary to a region of the gene outside of its region of homology with the donor sequences (nucleotides 5212883-5212905 on the "-" strand of chromosome 11), and a second primer complementary to a region of the donor molecule into which distinguishing mutations were introduced into the donor sequence (see *supra*). The sequences of these primers are given in Table 7 (labeled "chromosome-specific" and "donor-specific," respectively). Using these two primers, an amplification product of 415 base pairs will be obtained from genomic DNA if a targeted recombination event has occurred. As a control for DNA loading, PCR reactions were also carried out using the Initial amp 1 and Initial amp 2 primers to ensure that similar levels of genomic DNA were added to each PCR reaction. Conditions for amplification were: 95°C, 2 min, followed by 40 cycles of 95°C, 30 sec, 60°C, 45 sec, 68°C, 2 min; and a final step of 68°C, 10 min.

The results of this analysis (Figure 17) indicate that a 415 base pair amplification product was obtained only with DNA extracted from cells which had been transfected with the "high" concentration of donor plasmid and both ZFP-*FokI* plasmids, consistent with targeted recombination of donor sequences into the chromosomal beta-globin locus.

Table 7: Amplification Primers for the human beta globin gene

Initial amp 1	TACTGATGGTATGGGGCCAAGAG (SEQ ID NO:*)
Initial amp 2	CACGTGCAGCTTGTACAGTGC (SEQ ID NO:*)
Chromosome-specific	TGCTTACCAAGCTGTGATTC (SEQ ID NO:*)
Donor-specific	GGTTGACGATCGGAATTC (SEQ ID NO:*)

To confirm this result, the amplification product was cloned into pCR4-TOPO (Invitrogen) and its nucleotide sequence was determined. As shown in Figure 18 (SEQ

ID NO: **), the sequence consists of a fusion between chromosomal sequences not present on the donor plasmid and sequences unique to the donor plasmid. For example, two C→G mutations which disrupt ZFP-binding are observed at positions 377 and 383 in the sequence. Nucleotides 377-408 represent sequence obtained from the donor plasmid containing the sequence changes described above; nucleotides 73-376 are sequences common to the donor and the chromosome, and nucleotides 1-72 are unique to the chromosome. The covalent linkage of donor-specific and chromosome-specific sequences in the genome confirms the successful recombination of the donor sequence at the correct locus within the genome of K562 cells.

Example 4: ZFP-FokI linker (ZC linker) optimization

In order to test the effect of ZC linker length on cleavage efficiency, a four-finger ZFP binding domain was fused to a *FokI* cleavage half-domain, using ZC linkers of various lengths. The target site for the ZFP is 5'-AACTCGGATAAT-3' (SEQ ID NO:*) and the amino acid sequences of the recognition regions (positions -1 through +6 with respect to the start of the alpha-helix) of each of the zinc fingers were as follows (wherein F1 is the N-most, and F4 is the C-most zinc finger):

F1: DRSTLIE (SEQ ID NO:*)

F2: SSSNLSR (SEQ ID NO:*)

F3: RSDDLK (SEQ ID NO:*)

F4: DNSNRIK (SEQ ID NO:*)

ZFP-*FokI* fusions, in which the aforementioned ZFP binding domain and a *FokI* cleavage half-domain were separated by 2, 3, 4, 5, 6, or 10 amino acid residues, were constructed. Each of these proteins was tested for cleavage of substrates having an inverted repeat of the ZFP target site, with repeats separated by 4, 5, 6, 7, 8, 9, 12, 15, 16, 17, 22, or 26 basepairs.

The amino acid sequences of the fusion constructs, in the region of the ZFP-*FokI* junction (with the ZC linker sequence underlined), are as follows:

10-residue linker HTKIHLRQKDAARGSQLV (SEQ ID NO*)

6-residue linker HTKIHLRQKGSQVL (SEQ ID NO*)

5-residue linker HTKIHLRQGSQVL (SEQ ID NO*)

4-residue linker HTKIHLRGSQVLV (SEQ ID NO⑥)
3-residue linker HTKIHLGSQVLV (SEQ ID NO⑥)
2-residue linker HTKIHGSQVLV (SEQ ID NO⑥)

5 The sequences of the various cleavage substrates, with the ZFP target sites
underlined, are as follows:

4bp separation CTAGCATTATCCGAGTTACACAACTCGGATAATGCTAG
10 GATCGTAATAGGCTCAATGTGTGTGAGCCTATTACGATC
 (SEQ ID NO:*)

5bp separation CTAGCATTATCCGAGTTACACAACTCGGATAATGCTAG
 GATCGTAATAGGCTCAAGTGTGTGTGAGCCTATTACGATC
 (SEQ ID NO:*)

15 6bp separation CTAGGCATTATCCGAGTTCACCACAACCTCGGATAATGACTAG
 GATCCGTAATAGGCTCAAGTGGTGTGTGAGCCTATTACTGATC
 (SEQ ID NO:*)

20 7bp separation CTAGCATTATCCGAGTTCACACACAACCTCGGATAATGCTAG
 GATCGTAATAGGCTCAAGTGTGTGTGAGCCTATTACGATC
 (SEQ ID NO:*)

25 8bp separation CTAGCATTATCCGAGTTCACCACACAACCTCGGATAATGCTAG
 GATCGTAATAGGCTCAAGTGGTGTGTGTGAGCCTATTACGATC
 (SEQ ID NO:*)

30 9bp separation CTAGCATTATCCGAGTTCACACACAACAACCTCGGATAATGCTAG
 GATCGTAATAGGCTCAAGTGTGTGTGTGAGCCTATTACGATC
 (SEQ ID NO:*)

35 12bp separation CTAGCATTATCCGAGTTCACCACCAACACAACCTCGGATAATGCTAG
 GATCGTAATAGGCTCAAGTGGTGGTGTGTGTGAGCCTATTACGATC
 (SEQ ID NO:*)

40 15bp separation CTAGCATTATCCGAGTTCACCACCAACCACACAACCTCGGATAATGCTAG
 GATCGTAATAGGCTCAAGTGGTGGTGGTGTGTGTGAGCCTATTACGATC
 (SEQ ID NO:*)

45 16bp separation CTAGCATTATCCGAGTTCACCACCAACCACCAACCTCGGATAATGCTAG
 GATCGTAATAGGCTCAAGTGGTGGTGGTGTGTGTGTGAGCCTATTACGATC
 (SEQ ID NO:*)

50 17bp separation CTAGCATTATCCGAGTTCACCACCAACCACCAACCTCGGATAATGCTAG
 GATCGTAATAGGCTCAAGTGGTGGTGGTGTGTGTGTGTGAGCCTATTACGATC
 (SEQ ID NO:*)

22bp separation CTAGCATTATCCGAGTTCACCACCAACCACCAACACAACCTCGGATAATGCTAG
 GATCGTAATAGGCTCAAGTGGTGGTGGTGTGTGTGTGTGAGCCTATTACGATC
 (SEQ ID NO:*)

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25

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Table 8: DNA cleavage efficiency for various ZC linker lengths and various binding site separations*

	2-residue	3-residue	4-residue	5-residue	6-residue	10-residue
4 bp	74%	81%	74%	12%	6%	4%
5 bp	61%	89%	92%	80%	53%	40%
6 bp	78%	89%	95%	91%	93%	76%
7 bp	15%	55%	80%	80%	70%	80%
8 bp	0%	0%	8%	11%	22%	63%
9 bp	2%	6%	23%	9%	13%	51%
12 bp	8%	12%	22%	40%	69%	84%
15 bp	73%	78%	97%	92%	95%	88%
16 bp	59%	89%	100%	97%	90%	86%
17 bp	5%	22%	77%	71%	85%	82%
22 bp	1%	3%	5%	8%	18%	58%
26 bp	1%	2%	35%	36%	84%	78%

- 5 * The columns represent different ZFP-*FokI* fusion constructs with the indicated number of residues separating the ZFP and the *FokI* cleavage half-domain. The rows represent different DNA substrates with the indicated number of basepairs separating the inverted repeats of the ZFP target site.

- 10 For ZFP-*FokI* fusions with four residue linkers, the amino acid sequence of the linker was also varied. In separate constructs, the original LRGS linker sequence (SEQ ID NO:**) was changed to LGGS (SEQ ID NO:**), TGGS (SEQ ID NO:**), GGGS (SEQ ID NO:**), LPGS (SEQ ID NO:**), LRKS (SEQ ID NO:**), and LRWS (SEQ ID NO:**); and the resulting fusions were tested on substrates having a six-basepair separation between binding sites. Fusions containing the LGGS (SEQ ID NO: SAME AS ABOVE) linker sequence were observed to cleave more efficiently than those containing
- 15 the original LRGS sequence(SEQ ID NO: SAME AS ABOVE). Fusions containing the LRKS(SEQ ID NO: SAME AS ABOVE) and LRWS(SEQ ID NO: SAME AS ABOVE) sequences cleaved with less efficiency than the LRGS sequence(SEQ ID NO: SAME AS ABOVE), while the cleavage efficiencies of the remaining fusions were similar to that of the fusion comprising the original LRGS sequence(SEQ ID NO: SAME AS ABOVE).

20

Example 5: Increased cleavage specificity resulting from alteration of the *FokI* cleavage half-domain in the dimerization interface

A pair of ZFP/*FokI* fusion proteins (denoted 5-8 and 5-10) were designed to bind to target sites in the fifth exon of the IL-2R γ gene, to promote cleavage in the region

between the target sites. The relevant region of the gene, including the target sequences of the two fusion proteins, is shown in Figure 19. The amino acid sequence of the 5-8 protein is shown in Figure 20, and the amino acid sequence of the 5-10 protein is shown in Figure 21. With respect to the zinc finger portion of these proteins, the DNA target sequences, as well as amino acid sequences of the recognition regions in the zinc fingers, are given in Table 9.

Table 9: Zinc Finger Designs for the IL2Ry Gene

Fusion	Target sequence	F1	F2	F3	F4
5-8 (A)	ACTCTGTGGAAG (SEQ ID NO:**))	RSDNLSE (SEQ ID NO:**))	RNAHRIN (SEQ ID NO:**))	RSDTLSE (SEQ ID NO:**))	ARSTRIT (SEQ ID NO:**))
5-10 (B)	AACACGaAACGTG (SEQ ID NO:**))	RSDSLSR (SEQ ID NO:**))	DSSNRKT (SEQ ID NO:**))	RSDSLSV (SEQ ID NO:**))	DRSNRIT (SEQ ID NO:**))

Note: The zinc finger amino acid sequences shown above (in one-letter code) represent residues -1 through +6, with respect to the start of the alpha-helical portion of each zinc finger. Finger F1 is closest to the amino terminus of the protein.

The ability of this pair of fusion proteins to catalyze specific cleavage of DNA between their target sequences (see Figure 19) was tested *in vitro* using a labeled DNA template containing the target sequence and assaying for the presence of diagnostic digestion products. Specific cleavage was obtained when both proteins were used (Table 10, first row). However, the 5-10 fusion protein (comprising a wild-type *FokI* cleavage half-domain) was also capable of aberrant cleavage at a non-target site in the absence of the 5-8 protein (Table 10, second row), possibly due to self-dimerization.

Accordingly, 5-10 was modified in its *FokI* cleavage half-domain by converting amino acid residue 490 from glutamic acid (E) to lysine (K). (Numbering of amino acid residues in the *FokI* protein is according to Wah *et al., supra.*) This modification was designed to prevent homodimerization by altering an amino acid residue in the dimerization interface. The 5-10 (E490K) mutant, unlike the parental 5-10 protein, was unable to cleave at aberrant sites in the absence of the 5-8 fusion protein (Table 10, Row 3). However, the 5-10 (E490K) mutant, together with the 5-8 protein, catalyzed specific cleavage of the substrate (Table 10, Row 4). Thus, alteration of a residue in the cleavage

half-domain of 5-10, that is involved in dimerization, prevented aberrant cleavage by this fusion protein due to self-dimerization.

In addition, the 5-8 protein was modified in its dimerization interface by replacing the glutamine (Q) residue at position 486 with glutamic acid (E). This 5-8 (Q486E) mutant was tested for its ability to catalyze targeted cleavage in the presence of either the wild-type 5-10 protein or the 5-10 (E490K) mutant. DNA cleavage was not observed when the labeled substrate was incubated in the presence of both 5-8 (Q486E) and wild-type 5-10 (Table 10, Row 5). However, cleavage was obtained when the 5-8 (Q486E) and 5-10 (E490K) mutants were used in combination (Table 10, Row 6).

These results indicate that DNA cleavage by a ZFP/*FokI* fusion protein pair, at regions other than that defined by the target sequences of the two fusion proteins, can be minimized or abolished by altering the amino acid sequence of the cleavage half-domain in one or both of the fusion proteins.

Table 10: DNA cleavage by ZFP/*FokI* fusion protein pairs containing wild-type and mutant cleavage half-domains

	ZFP 5-8 binding domain	ZFP 5-10 binding domain	DNA cleavage
1	Wild-type <i>FokI</i>	Wild-type <i>FokI</i>	Specific
2	Not present	Wild-type <i>FokI</i>	Non-specific
3	Not present	<i>FokI</i> E490K	None
4	Wild-type <i>FokI</i>	<i>FokI</i> E490K	Specific
5	<i>FokI</i> Q486E	Wild-type	None
6	<i>FokI</i> Q486E	<i>FokI</i> E490K	Specific

Note: Each row of the table presents results of a separate experiment in which ZFP/*FokI* fusion proteins were tested for cleavage of a labeled DNA substrate. One of the fusion proteins contained the 5-8 DNA binding domain, and the other fusion protein contained the 5-10 DNA binding domain (See Table 9 and Figure 19). The cleavage half-domain portion of the fusion proteins was as indicated in the Table. Thus, the entries in the ZFP 5-8 column indicate the type of *FokI* cleavage domain fused to ZFP 5-8; and the entries in the ZFP 5-10 column indicates the type of *FokI* cleavage domain fused to ZFP 5-10. For the *FokI* cleavage half-domain mutants, the number refers to the amino acid residue in the *FokI* protein; the letter preceding the number refers to the amino acid present in the wild-type protein and the letter following the number denotes the amino acid to which the wild-type residue was changed in generating the modified protein. 'Not present' indicates that the entire ZFP/*FokI* fusion protein was omitted from that particular experiment.

The DNA substrate used in this experiment was an approximately 400 bp PCR product containing the target sites for both ZFP 5-8 and ZFP 5-10. See Figure 19 for the sequences and relative orientation of the two target sites.

Example 6: Generation of a defective enhanced Green Fluorescent Protein (eGFP) gene

The enhanced Green Fluorescent Protein (eGFP) is a modified form of the Green Fluorescent Protein (GFP; *see, e.g., Tsien (1998) Ann. Rev. Biochem. 67:509-544*) containing changes at amino acid 64 (phe to leu) and 65 (ser to thr). Heim *et al.* (1995) *Nature* 373:663-664; Cormack *et al.* (1996) *Gene* 173:33-38. An eGFP-based reporter system was constructed by generating a defective form of the eGFP gene, which contained a stop codon and a 2-bp frameshift mutation. The sequence of the eGFP gene is shown in Figure 22. The mutations were inserted by overlapping PCR mutagenesis, using the Platinum® *Taq* DNA Polymerase High Fidelity kit (Invitrogen) and the oligonucleotides GFP-Bam, GFP-Xba, stop sense2, and stop anti2 as primers (oligonucleotide sequences are listed below in Table 11). GFP-Bam and GFP-Xba served as the external primers, while the primers stop sense2 and stop anti2 served as the internal primers encoding the nucleotide changes. The peGFP-N1 vector (BD Biosciences), encoding a full-length eGFP gene, was used as the DNA template in two separate amplification reactions, the first utilizing the GFP-Bam and stop anti2 oligonucleotides as primers and the second using the GFP-Xba and stop sense2 oligonucleotides as primers. This generated two amplification products whose sequences overlapped. These products were combined and used as template in a third amplification reaction, using the external GFP-Bam and GFP-Xba oligonucleotides as primers, to regenerate a modified eGFP gene in which the sequence GACCACAT (SEQ ID NO: **) at nucleotides 280-287 was replaced with the sequence TAACAC (SEQ ID NO: **). The PCR conditions for all amplification reactions were as follows: the template was initially denatured for 2 minutes at 94 degrees and followed by 25 cycles of amplification by incubating the reaction for 30 sec. at 94 degrees C, 45 sec. at 46 degrees C, and 60 sec. at 68 degrees C. A final round of extension was carried out at 68 degrees C for 10 minutes. The sequence of the final amplification product is shown in Figure 23. This 795 bp fragment was cloned into the pCR(R)4-TOPO vector using the TOPO-TA cloning kit (Invitrogen) to generate the pCR(R)4-TOPO-GFPmut construct.

Table 11: Oligonucleotide sequences for GFP

Oligo	sequence 5'-3'
GFP-Bam	CGAATTCTGCAGTCGAC (SEQ ID NO: **)
GFP-Xba	GATTATGATCTAGAGTCG (SEQ ID NO: **)
stop sense2	AGCCGCTACCCCTAACACGAAGCAG (SEQ ID NO: **)
stop anti2	CTGCTTCGTGTTAGGGGTAGCGGCT (SEQ ID NO: **)

Example 7: Design and assembly of Zinc Finger Nucleases targeting eGFP

Two three-finger ZFPs were designed to bind a region of the mutated GFP gene (Example 6) corresponding to nucleotides 271-294 (numbering according to Figure 23). The binding sites for these proteins occur in opposite orientation with 6 base pairs separating the two binding sites. See Figure 23. ZFP 287A binds nucleotides 271-279 on the non-coding strand, while ZFP 296 binds nucleotides 286-294 on the coding strand. The DNA target and amino acid sequence for the recognition regions of the ZFPs are listed below, and in Table 12:

287A:

F1 (GCGg) RSDDLTR (SEQ ID NO: **)
F2 (GTA) QSGALAR (SEQ ID NO: **)
F3 (GGG) RSDHLR (SEQ ID NO: **)

296S:

F1 (GCA) QSGSLTR (SEQ ID NO: **)
F2 (GCA) QSGDLTR (SEQ ID NO: **)
F3 (GAA) QSGNLAR (SEQ ID NO: **)

Table 12: Zinc finger designs for the GFP gene

Protein	Target sequence	F1	F2	F3
287A	GGGGTAGCGg (SEQ ID NO: **)	RSDDLTR (SEQ ID NO: **)	QSGALAR (SEQ ID NO: **)	RSDHLR (SEQ ID NO: **)
296S	GAAGCAGCA (SEQ ID NO: **)	QSGSLTR (SEQ ID NO: **)	QSGDLTR (SEQ ID NO: **)	QSGNLAR (SEQ ID NO: **)

Note: The zinc finger amino acid sequences shown above (in one-letter code) represent residues -1 through +6, with respect to the start of the alpha-helical portion of each zinc finger. Finger F1 is closest to the amino terminus of the protein, and Finger F3 is closest to the carboxy terminus.

Sequences encoding these proteins were generated by PCR assembly (e.g., U.S. Patent No. 6,534,261), cloned between the *KpnI* and *BamHI* sites of the pcDNA3.1

vector (Invitrogen), and fused in frame with the catalytic domain of the *FokI* endonuclease (amino acids 384-579 of the sequence of Looney *et al.* (1989) *Gene* 80:193-208). The resulting constructs were named pcDNA3.1-GFP287-FokI and pcDNA3.1-GFP296-FokI (Figure 24).

Example 8: Targeted *in vitro* DNA cleavage by designed Zinc Finger Nucleases

The pCR(R)4-TOPO-GFPmut construct (Example 6) was used to provide a template for testing the ability of the 287 and 296 zinc finger proteins to specifically recognize their target sites and cleave this modified form of eGFP *in vitro*.

A DNA fragment containing the defective eGFP-encoding insert was obtained by PCR amplification, using the T7 and T3 universal primers and pCR(R)4-TOPO-GFPmut as template. This fragment was end-labeled using γ -³²P-ATP and T4 polynucleotide kinase. Unincorporated nucleotide was removed using a microspin G-50 column (Amersham).

An *in vitro* coupled transcription/translation system was used to express the 287 and 296 zinc finger nucleases described in Example 7. For each construct, 200 ng linearized plasmid DNA was incubated in 20 μ L TnT mix and incubated at 30° C for 1 hour and 45 minutes. TnT mix contains 100 μ L TnT lysate (which includes T7 RNA polymerase, Promega, Madison, WI) supplemented with 2 μ L Methionine (1 mM) and 2.5 μ L ZnCl₂ (20 mM).

For analysis of DNA cleavage, aliquots from each of the 287 and 296 coupled transcription/translation reaction mixtures were combined, then serially diluted with cleavage buffer. Cleavage buffer contains 20 mM Tris-HCl pH 8.5, 75 mM NaCl, 10 mM MgCl₂, 10 μ M ZnCl₂, 1 mM DTT, 5% glycerol, 500 μ g/ml BSA. 5 μ L of each dilution was combined with approximately 1 ng DNA substrate (end-labeled with ³²P using T4 polynucleotide kinase as described above), and each mixture was further diluted to generate a 20 μ L cleavage reaction having the following composition: 20 mM Tris-HCl pH 8.5, 75 mM NaCl, 10 mM MgCl₂, 10 μ M ZnCl₂, 1 mM DTT, 5% glycerol, 500 μ g/ml BSA. Cleavage reactions were incubated for 1 hour at 37°C. Protein was extracted by adding 10 μ L phenol-chloroform solution to each reaction, mixing, and centrifuging to

separate the phases. Ten microliters of the aqueous phase from each reaction was analyzed by electrophoresis on a 10% polyacrylamide gel.

The gel was subjected to autoradiography, and the results of this experiment are shown in Figure 25. The four left-most lanes show the results of reactions in which the final dilution of each coupled transcription/translation reaction mixture (in the cleavage reaction) was 1/156.25, 1/31.25, 1/12.5 and 1/5, respectively, resulting in effective volumes of 0.032, 0.16, 0.4, and 1 μ l, respectively of each coupled transcription/translation reaction. The appearance of two DNA fragments having lower molecular weights than the starting fragment (lane labeled "uncut control" in Figure 25) is correlated with increasing amounts of the 287 and 296 zinc finger endonucleases in the reaction mixture, showing that DNA cleavage at the expected target site was obtained.

Example 9: Generation of stable cell lines containing an integrated defective eGFP gene

A DNA fragment encoding the mutated eGFP, eGFPmut, was cleaved out of the pCR(R)4-TOPO-GFPmut vector (Example 6) and cloned into the *HindIII* and *NorI* sites of pcDNA4/TO, thereby placing this gene under control of a tetracycline-inducible CMV promoter. The resulting plasmid was named pcDNA4/TO/GFPmut (Figure 26). T-Rex 293 cells (Invitrogen) were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% Tet-free fetal bovine serum (FBS) (HyClone). Cells were plated into a 6-well dish at 50% confluence, and two wells were each transfected with pcDNA4/TO/GFPmut. The cells were allowed to recover for 48 hours, then cells from both wells were combined and split into 10x15-cm² dishes in selective medium, *i.e.*, medium supplemented with 400 μ g/ml Zeocin (Invitrogen). The medium was changed every 3 days, and after 10 days single colonies were isolated and expanded further. Each clonal line was tested individually for doxycycline(dox)-inducible expression of the eGFPmut gene by quantitative RT-PCR (TaqMan[®]).

For quantitative RT-PCR analysis, total RNA was isolated from dox-treated and untreated cells using the High Pure Isolation Kit (Roche Molecular Biochemicals), and 25 ng of total RNA from each sample was subjected to real time quantitative RT-PCR to analyze endogenous gene expression, using TaqMan[®] assays. Probe and primer

sequences are shown in Table 13. Reactions were carried out on an ABI 7700 SDS machine (PerkinElmer Life Sciences) under the following conditions. The reverse transcription reaction was performed at 48° C for 30 minutes with MultiScribe reverse transcriptase (PerkinElmer Life Sciences), followed by a 10-minute denaturation step at 95° C. Polymerase chain reaction (PCR) was carried out with AmpliGold DNA polymerase (PerkinElmer Life Sciences) for 40 cycles at 95° C for 15 seconds and 60° C for 1 minute. Results were analyzed using the SDS version 1.7 software and are shown in Figure 27, with expression of the eGFPmut gene normalized to the expression of the human GAPDH gene. A number of cell lines exhibited doxycycline-dependent expression of eGFP; line 18 (T18) was chosen as a model cell line for further studies.

Table 13: Oligonucleotides for mRNA analysis

Oligonucleotide	Sequence
eGFP primer 1 (5T)	CTGCTGCCCCGACAACCA (SEQ ID NO: **)
eGFP primer 2 (3T)	CCATGTGATCGCGCTTCTC (SEQ ID NO: **)
eGFP probe	CCCAGTCCGCCCTGAGCAAAGA (SEQ ID NO: **)
GAPDH primer 1	CCATGTTTCGTCATGGGTGTGA (SEQ ID NO: **)
GAPDH primer 2	CATGGACTGTGGTCATGAGT (SEQ ID NO: **)
GAPDH probe	TCCTGCACCACCAACTGCTTAGCA (SEQ ID NO: **)

Example 10: Generation of a donor sequence for correction of a defective chromosomal eGFP gene

A donor construct containing the genetic information for correcting the defective eGFPmut gene was constructed by PCR. The PCR reaction was carried out as described above, using the pGFP-NI vector as the template. To prevent background expression of the donor construct in targeted recombination experiments, the first 12 bp and start codon were removed from the donor by PCR using the primers GFPnstart and GFP-Xba (sequences provided in Table 14). The resulting PCR fragment (734 bp) was cloned into the pCR(R)4-TOPO vector, which does not contain a mammalian cell promoter, by TOPO-TA cloning to create pCR(R)4-TOPO-GFPdonor5 (Figure 28). The sequence of

the eGFP insert of this construct (corresponding to nucleotides 64-797 of the sequence shown in Figure 22) is shown in Figure 29 (SEQ ID NO: **).

Table 14: Oligonucleotides for construction of donor molecule

Oligonucleotide	Sequence 5'-3'
GFPnostart	GGCGAGGAGCTGTCAC (SEQ ID NO: ***)
GFP-Xba	GATTATGATCTAGAGTCG (SEQ ID NO: ***)

Example 11: Correction of a mutation in an integrated chromosomal eGFP gene by targeted cleavage and recombination

The T18 stable cell line (Example 9) was transfected with one or both of the ZFP-FokI expression plasmid (pcDNA3.1-GFP287-FokI and pcDNA3.1-GFP296-FokI, Example 7) and 300 ng of the donor plasmid pCR(R)4-TOPO-GFPdonor5 (Example 10) using LipofectAMINE 2000 Reagent (Invitrogen) in Opti-MEM I reduced serum medium, according to the manufacturer's protocol. Expression of the defective chromosomal eGFP gene was induced 5-6 hours after transfection by the addition of 2 ng/ml doxycycline to the culture medium. The cells were arrested in the G2 phase of the cell cycle by the addition, at 24 hours post-transfection, of 100 ng/ml Nocodazole (Figure 30) or 0.2 uM Vinblastine (Figure 31). G2 arrest was allowed to continue for 24-48 hours, and was then released by the removal of the medium. The cells were washed with PBS and the medium was replaced with DMEM containing tetracycline-free FBS and 2 ng/ml doxycycline. The cells were allowed to recover for 24-48 hours, and gene correction efficiency was measured by monitoring the number of cells exhibiting eGFP fluorescence, by fluorescence-activated cell sorting (FACS) analysis. FACS analysis was carried out using a Beckman-Coulter EPICS XL-MCL instrument and System II Data Acquisition and Display software, version 2.0. eGFP fluorescence was detected by excitation at 488 nm with an argon laser and monitoring emissions at 525 nm (x-axis). Background or autofluorescence was measured by monitoring emissions at 570 nm (y-axis). Cells exhibiting high fluorescent emission at 525 nm and low emission at 570 nm (region E) were scored positive for gene correction.

The results are summarized in Table 15 and Figures 30 and 31. Figures 30 and 31 show results in which T18 cells were transfected with the pcDNA3.1-GFP287-FokI and pcDNA3.1-GFP296-FokI plasmids encoding ZFP nucleases and the pCR(R)4-TOPO-GFPdonor5 plasmid, eGFP expression was induced with doxycycline, and cells were
5 arrested in G2 with either nocodazole (Figure 30) or vinblastine (Figure 31). Both figures show FACS traces, in which cells exhibiting eGFP fluorescence are represented in the lower right-hand portion of the trace (identified as Region E, which is the portion of Quadrant 4 underneath the curve). For transfected cells that had been treated with nocodazole, 5.35% of the cells exhibited GFP fluorescence, indicative of correction of
10 the mutant chromosomal eGFP gene (Figure 30), while 6.7% of cells treated with vinblastine underwent eGFP gene correction (Figure 31). These results are summarized, along with additional control experiments, in Rows 1-8 of Table 15.

In summary, these experiments show that, in the presence of two ZFP nucleases and a donor sequence, approximately 1% of treated cells underwent gene correction, and
15 that this level of correction was increased 4-5 fold by arresting treated cells in the G2 phase of the cell cycle.

Table 15: Correction of a defective chromosomal eGFP gene

Expt.	Treatment ¹	Percent cells with corrected eGFP gene ²
1	300 ng donor only	0.01
2	100 ng ZFP 287 + 300 ng donor	0.16
3	100 ng ZFP 296 + 300 ng donor	0.6
4	50 ng ZFP 287 + 50 ng ZFP 296 + 300 ng donor	1.2
5	as 4 + 100 ng/ml nocodazole	5.35
6	as 4 + 0.2 uM vinblastine	6.7
7	no donor, no ZFP, 100 ng/ml nocodazole	0.01
8	no donor, no ZFP, 0.2 uM vinblastine	0.0
9	100 ng ZFP287/Q486E + 300 ng donor	0.0
10	100 ng ZFP296/E490K + 300 ng donor	0.01
11	50 ng 287/Q486E + 50 ng 296/E490K + 300 ng donor	0.62
12	as 11 + 100 ng/ml nocodazole	2.37
13	as 11 + 0.2 uM vinblastine	2.56

Notes:

1: T18 cells, containing a defective chromosomal eGFP gene, were transfected with plasmids encoding one or two ZFP nucleases and/or a donor plasmid encoding a nondefective eGFP sequence, and expression of the chromosomal eGFP gene was induced with doxycycline. Cells were optionally arrested in G2 phase of the cell cycle after eGFP induction. FACS analysis was conducted 5 days after transfection.

2: The number is the percent of total fluorescence exhibiting high emission at 525 nm and low emission at 570 nm (region E of the FACS trace).

Example 12: Correction of a defective chromosomal gene using zinc finger nucleases with sequence alterations in the dimerization interface

Zinc finger nucleases whose sequences had been altered in the dimerization interface were tested for their ability to catalyze correction of a defective chromosomal eGFP gene. The protocol described in Example 11 was used, except that the nuclease portion of the ZFP nucleases (*i.e.*, the *FokI* cleavage half-domains) were altered as described in Example 5. Thus, an E490K cleavage half-domain was fused to the GFP296 ZFP domain (Table 12), and a Q486E cleavage half-domain was fused to the GFP287 ZFP (Table 12).

The results are shown in Rows 9-11 of Table 15 and indicate that a significant increase in the frequency of gene correction was obtained in the presence of two ZFP nucleases having alterations in their dimerization interfaces, compared to that obtained in the presence of either of the nucleases alone. Additional experiments, in which T18 cells

were transfected with donor plasmid and plasmids encoding the 287/Q486E and 296/E490K zinc finger nucleases, then arrested in G2 with nocodazole or vinblastine, showed a further increase in frequency of gene correction, with over 2% of cells exhibiting eGFP fluorescence, indicative of a corrected chromosomal eGFP gene (Table 15, Rows 12 and 13).

Example 13: Effect of donor length on frequency of gene correction

In an experiment similar to those described in Example 11, the effect of the length of donor sequence on frequency of targeted recombination was tested. T18 cells were transfected with the two ZFP nucleases, and eGFP expression was induced with doxycycline, as in Example 11. Cells were also transfected with either the pCR(R)4-TOPO-GFPdonor5 plasmid (Figure 28) containing a 734 bp eGFP insert (Figure 29) as in Example 11, or a similar plasmid containing a 1527 bp sequence insert (Figure 32) homologous to the mutated chromosomal eGFP gene. Additionally, the effect of G2 arrest with nocodazole on recombination frequency was assessed.

The results, shown in Table 16, show that longer donor sequence increases the frequency of targeted recombination (and, hence, of gene correction) and confirm that arrest of cells in the G2 phase of the cell cycle also increases the frequency of targeted recombination.

Table 16: Effect of donor length and cell-cycle arrest on targeted recombination frequency

Donor length (kb)	Nocodazole concentration:	
	0 ng/ml	100 ng/ml
0.7	1.41	5.84
1.5	2.16	8.38

Note: Numbers represent percentage of total fluorescence in Region E of the FACS trace (see Example 11) which is an indication of the fraction of cells that have undergone targeted recombination to correct the defective chromosomal eGFP gene.

Example 14: Editing of an endogenous human IL-2R γ gene by targeted cleavage and recombination using zinc finger nucleases

Two expression vectors, each encoding a ZFP-nuclease targeted to the human IL-2R γ gene, were constructed. Each ZFP-nuclease contained a zinc finger protein-based

DNA binding domain (see Table 17) fused to the nuclease domain of the type IIS restriction enzyme *FokI* (amino acids 384-579 of the sequence of Wah *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:10564-10569) via a four amino acid ZC linker (see Example 4). The nucleases were designed to bind to positions in exon 5 of the chromosomal IL-2R γ gene surrounding codons 228 and 229, and to introduce a double-strand break in the DNA between their binding sites.

Table 17: Zinc Finger Designs for exon 5 of the IL2R γ Gene

Target sequence	F1	F2	F3	F4
ACTCTGTGGAAG (SEQ ID NO:)	RSDNLSV (SEQ ID NO:)	RNAHRIN (SEQ ID NO:)	RSDTLSE (SEQ ID NO:)	ARSTRTN (SEQ ID NO:)
AAAGCGGCTCCG (SEQ ID NO:)	RSDTLSE (SEQ ID NO:)	ARSTRIT (SEQ ID NO:)	RSDSLSK (SEQ ID NO:)	QRSNLKV (SEQ ID NO:)

Note: The zinc finger amino acid sequences shown above (in one-letter code) represent residues -1 through +6, with respect to the start of the alpha-helical portion of each zinc finger. Finger F1 is closest to the amino terminus of the protein.

The complete DNA-binding portion of each of the chimeric endonucleases was as follows:

Nuclease targeted to ACTCTGTGGAAG (SEQ ID NO: **SAME AS ABOVE**)

MAERPFQCRICMRNFSRSDNLSVHIRTHTGEKPFACDICGRKFARNHRIN
HTKIHTGSQKPFQCRICMRNFSRSDTLSEHIRTHTGEKPFACDICGRKFAARSTR
NHTKIHLRGS (SEQ ID NO: ***)

Nuclease targeted to AAAGCGGCTCCG (SEQ ID NO: **SAME AS ABOVE**)

MAERPFQCRICMRNFSRSDTLSEHIRTHTGEKPFACDICGRKFAARSTRTHTKIH
TGSQKPFQCRICMRNFSRSDSLSKHIRTHTGEKPFACDICGRKFAQRSNLKVHTKI
HLRGS (SEQ ID NO: ***)

Human embryonic kidney 293 cells were transfected (Lipofectamine 2000; Invitrogen) with two expression constructs, each encoding one of the ZFP-nucleases described in the preceding paragraph. The cells were also transfected with a donor construct carrying as an insert a 1,543 bp fragment of the IL2R γ locus corresponding to positions 69195166-69196708 of the "minus" strand of the X chromosome (UCSC

human genome release July 2003), in the pCR4Blunt Topo (Invitrogen) vector. The IL-2R γ insert sequence contained the following two point mutations in the sequence of exon 5 (underlined):

F R V R S R F N P L C G S (SEQ ID NO: ***)
5 TTTCGTGTTCGGAGCCGGTTTAACCCGCTCTGTGGAAGT (SEQ ID NO: ***)

The first mutation (CGC→CGG) does not change the amino acid sequence and serves to adversely affect the ability of the ZFP-nuclease to bind to the donor DNA, and to chromosomal DNA following recombination. The second mutation (CCA→CCG) does not change the amino acid sequence and creates a recognition site for the restriction enzyme *Bsr*BI.

10 Either 50 or 100 nanograms of each ZFP-nuclease expression construct and 0.5 or 1 microgram of the donor construct were used in duplicate transfections. The following control experiments were also performed: transfection with an expression plasmid encoding the eGFP protein; transfection with donor construct only; and transfection with plasmids expressing the ZFP nucleases only. Twenty four hours after transfection,
15 vinblastine (Sigma) was added to 0.2 μ M final concentration to one sample in each set of duplicates, while the other remained untreated. Vinblastine affects the cell's ability to assemble the mitotic spindle and therefore acts as a potent G₂ arresting agent. This treatment was performed to enhance the frequency of targeting because the homology-directed double-stranded break repair pathway is more active than non-homologous end-
20 joining in the G₂ phase of the cell cycle. Following a 48 hr period of treatment with 0.2 μ M vinblastine, growth medium was replaced, and the cells were allowed to recover from vinblastine treatment for an additional 24 hours. Genomic DNA was then isolated from all cell samples using the DNEasy Tissue Kit (Qiagen). Five hundred nanograms of
25 genomic DNA from each sample was then assayed for frequency of gene targeting, by testing for the presence of a new *Bsr*BI site in the chromosomal IL-2R γ locus, using the assay described schematically in Figure 33.

In brief, 20 cycles of PCR were performed using the primers shown in Table 18, each of which hybridizes to the chromosomal IL-2R γ locus immediately outside of the region homologous to the 1.5 kb donor sequence. Twenty microcuries each of α -³²P-dCTP and α -³²P-dATP were included in each PCR reaction to allow detection of PCR
30

products. The PCR reactions were desalted on a G-50 column (Amersham), and digested for 1 hour with 10 units of *Bsr*BI (New England Biolabs). The digestion products were resolved on a 10% non-denaturing polyacrylamide gel (BioRad), and the gel was dried and autoradiographed (Figure 34). In addition to the major PCR product, corresponding to the 1.55 kb amplified fragment of the IL2Ry locus ("wt" in Figure 34), an additional band ("rflp" in Figure 34) was observed in lanes corresponding to samples from cells that were transfected with the donor DNA construct and both ZFP-nuclease constructs. This additional band did not appear in any of the control lanes, indicating that ZFP nuclease-facilitated recombination of the *Bsr*BI RFLP-containing donor sequence into the chromosome occurred in this experiment.

Additional experiments, in which trace amounts of a RFLP-containing IL-2Ry DNA sequence was added to human genomic DNA (containing the wild-type IL-2Ry gene), and the resultant mixture was amplified and subjected to digestion with a restriction enzyme which cleaves at the RFLP, have indicated that as little as 0.5% RFLP-containing sequence can be detected quantitatively using this assay.

Table 18: Oligonucleotides for analysis of the human IL-2Ry gene

Oligonucleotide	Sequence
Ex5_1.5detF1	GATTCAACCAGACAGATAGAAGG (SEQ ID NO: ***)
Ex5_1.5detR1	TTACTGTCTCATCCTTTACTCC (SEQ ID NO: ***)

All patents, patent applications and publications mentioned herein are hereby incorporated by reference, in their entireties, for all purposes.

Although disclosure has been provided in some detail by way of illustration and example for the purposes of clarity of understanding, it will be apparent to those skilled in the art that various changes and modifications can be practiced without departing from the spirit or scope of the disclosure. Accordingly, the foregoing descriptions and examples should not be construed as limiting.

**METHODS AND COMPOSITIONS FOR TARGETED CLEAVAGE
AND RECOMBINATION**

ABSTRACT OF THE DISCLOSURE

5 Disclosed herein are methods and compositions for targeted cleavage of a genomic sequence, and targeted recombination between a genomic region and an exogenous polynucleotide homologous to the genomic region.

10 The compositions include fusion proteins comprising a cleavage domain (or cleavage half-domain) and an engineered zinc finger domain and polynucleotides encoding same. Methods for targeted cleavage include introduction of such fusion proteins, or polynucleotides encoding same, into a cell. Methods for targeted recombination additionally include introduction of an exogenous polynucleotide homologous to a genomic region into cells comprising the disclosed fusion proteins.

FIGURE 1

MetGlyPheLeuLysLeuIle
CTGCCGCCGGCGCCGGCCGTCATGGGGTTCCTGAAACTGATT
GACGGCGGCCCGCGGCCGGCAGTACCCCAAGGACTTTGACTAA

5 10 15 20 25 30 35 40

(SEQ ID NO: **)

FIGURE 2

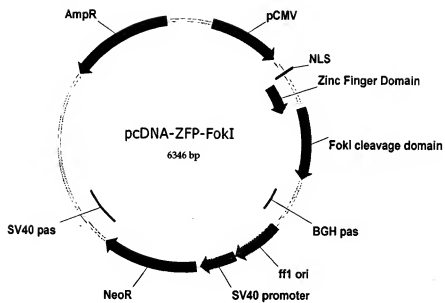


FIGURE 3

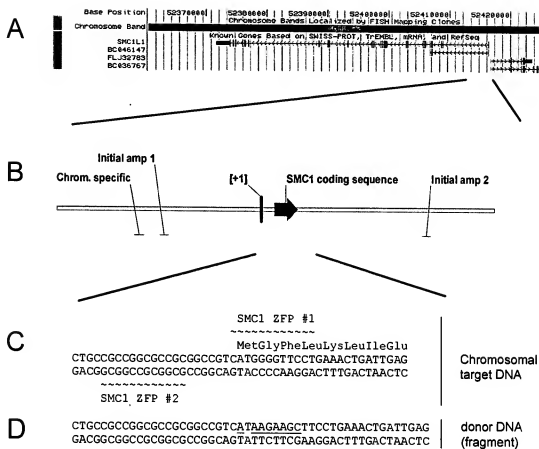


FIGURE 4

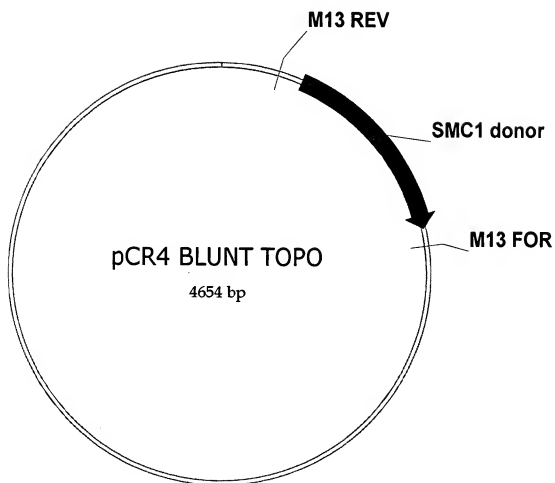


FIGURE 5

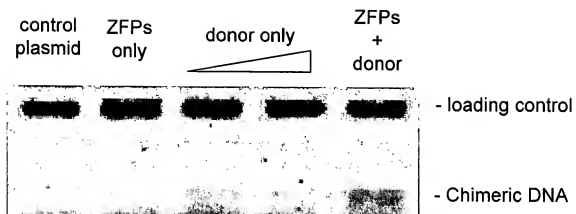


FIGURE 6

<u>TAGTCCTGCAGGTTTAAACGAATTCGCCCTTCTCAGCAAGCGTGAGCTCA</u>	50
<u>GGTCTCCCCCGCTCCTTGAACCTCAAGAACTGCTCTGACTCCGCCAGC</u>	100
<u>AACAACTCCTCCGGGATCTGGTCCGCAGGAGCAAGTGTTTGTTGTGCC</u>	150
<u>ATGCAACAAGAAAAGGGGCGGAGGCACACGCCAGTCGTCAGCTCGCTC</u>	200
<u>CTCGTATACGCAACATCAGTCCCCGCCCTGGTCCCACTCCTGCCGGAAG</u>	250
<u>GCGAAGATCCCGTTAGGCCTGGACGTATTCTCGCGACATTGCGGTCGC</u>	300
<u>CCGGCTTGCACTGCGGCGTTTCCCGCGCGGGCTACCTCAGTTCTCGGGCG</u>	350
<u>TACGGCGCGGCCTGTCTACTGCTGCCGCGCGCGGCCGTCATaagaag</u>	400
<u>cTTCCTGAAACTGATTGAAGGGCGAATTCGCGGCCGCTAAATTCATTTCG</u>	450
<u>CCCTATAGTGAGT</u>	

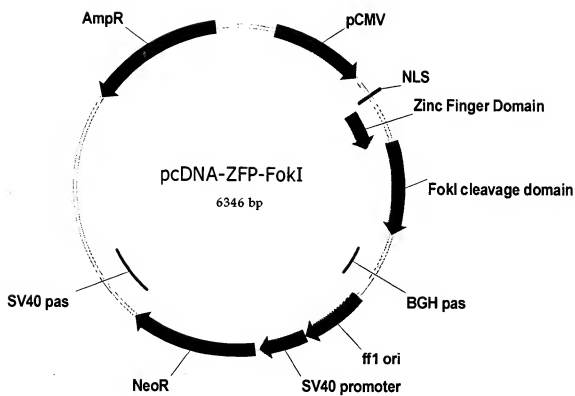
(SEQ ID NO: **)

FIGURE 7

TyrLysAsnSerAspAsnAspLysVal
CTTCCAACCTTTCTCCTCTAGGTACAAGAACTCGGATAATGATAAAGTCC
GAAGGTTGGAAAGAGGAGATCCATGTTCTTGAGCCTATTACTATTTCAGG
| | | | | | | | | | | | | | | | | | | | | | | | | |
5 10 15 20 25 30 35 40 45 50

(SEQ ID NO: **)

FIGURE 8



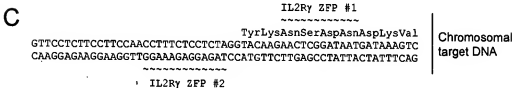
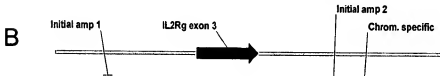


FIGURE 10

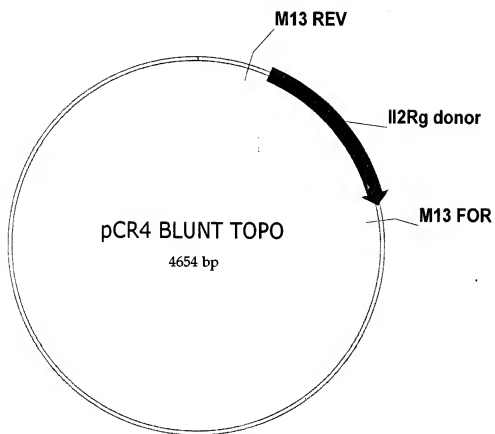


FIGURE 11

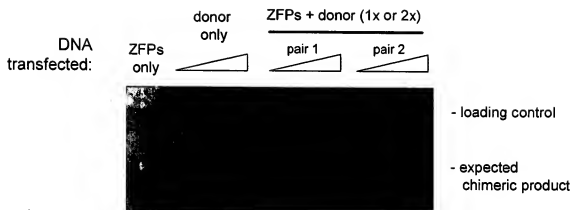


FIGURE 12

<u>TAGTCCTGCAGGTTTAAACGAATTCGCCCTTTCCTCTAGGTA</u>	50
<u>CcGAcAAcGATAAAGTCCAGAAGTGCAGCCACTATCTATTCCCTGAAGAA</u>	100
ATCACTTCTGGCTGTCAGTTGCAAAAAAAGGAGATCCACCTCTACCAAAAC	150
ATTTGTTGTTTCAGCTCCAGGACCCACGGGAACCCAGGAGACAGGCCACAC	200
AGATGCTAAAACTGCAGAATCTGGGTAATTTGGAAAGAAAGGGTCAAGAG	250
ACCAGGGTACTGTGGGACATTGGAGTCTACAGAGTAGTGTCTTTTATC	300
ATAAGGGTACATGGGCAGAAAAGAGGAGGTAGGGGATCATGATGGGAAGG	350
GAGGAGGTATTAGGGGCACTACCTTCAGGATCCTGACTTGTCTAGGCCAG	400
GGGAATGACCACATATGCACACATATCTCCAGTGATCCCCTGGGCTCCAG	450
AGAACCTAACACTTCACAAACTGAGTGAATCCCAGCTAGAACTGAACTGG	500
<u>AACAACAGATTCTTGAACCACTGTTTGGAGCACTTGGTGAGTACCGGAC</u>	550
<u>TAAGGGCGAATTCGCGGCCGCTAAATTCAATTCGCCCTATAGTGAGTCGT</u>	600
<u>ATTACAATTCACTGGCCGTCGTTT</u>	

(SEQ ID NO: **)

FIGURE 13

TACTGATGGTATGGGGCCAAGAGATATATCTTAGAGGGAGGGCTGAGGGT	50
TTGAAGTCCAACCTCCTAAGCCAGTGCCAGAAGAGCCAAGGACAGGTACGG	100
CTGTCACTACTTAGACCTCACCCCTGTGGAGCCACACCCTAGGGTTGGCCA	150
ATCTACTCCCAGGAGCAGGGAGGGCAGGAGCCAGGGCTGGGCATAAAAGT	200
CAGGGCAGAGCCATCTATTGCTTACATTGCTTCTGACACAACCTGTGTTC	250
ACTAGCAACCTCAAACAGACACCATGGTGCATCTGACTCCTGAGGAGAAG	300
TCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGG	350
TGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGA	400
CCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGAT	450
AGGCACCTGACTCTCTCTGCCTATTGGTCTATTTCCACCCCTTAGGCTGC	500
TGGTGGTCTACCCCTTGGAACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTG	550
TCCACTCCTGATGCTGTATATGGGCAACCCTAAGGTGAAGGCTCATGGCAA	600
GAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCA	650
AGGGCACCTTTGCCACACTGAGTGAGCTGACTGTGACAAGCTGCACGTG	700

(SEQ ID NO: **)

FIGURE 14

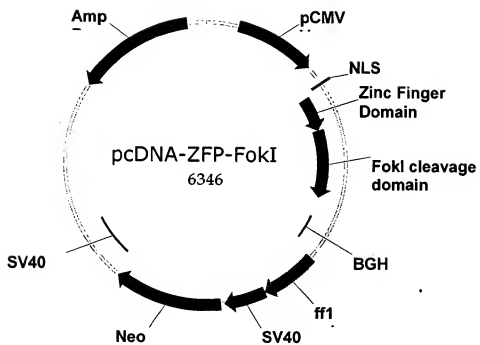


FIGURE 15

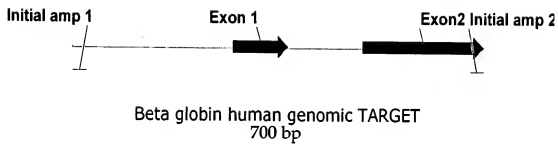


FIGURE 16

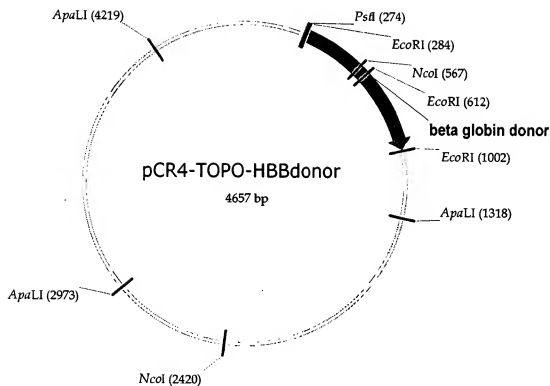


FIGURE 17

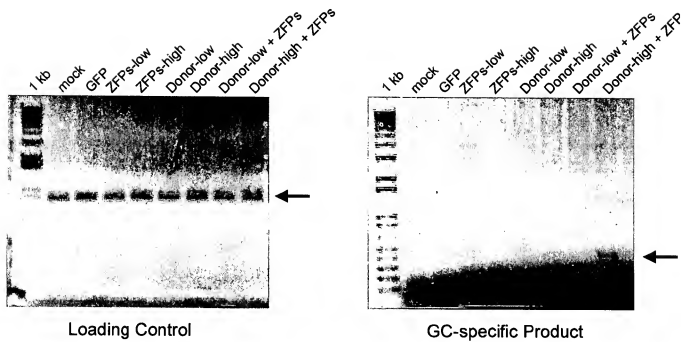


FIGURE 18

TGCTTACCAAGCTGTGATTCCAAATATTACGTAAATACACTTGCAAAGGA	50
GGATGTTTTTTAGTAGCAATTTGTACTGATGGTATGGGGCCAAGAGATATA	100
TCTTAGAGGGAGGGCTGAGGGTTTGAAGTCCAACCTCTAAGCCAGTGCCA	150
GAAGAGCCAAGGACAGGTACGGCTGTCATCACTTAGACCTCACCTGTGG	200
AGCCACACCCTAGGGTTGGCCAATCTACTCCCAGGAGCAGGGAGGGCAGG	250
AGCCAGGGCTGGGCATAAAAGTCAGGGCAGAGCCATCTATTGCTTACATT	300
TGCTTCTGACACAACCTGTGTCTACTAGCAACCTCAAACAGACACCATGGT	350
GCATCTGACTCCTGAGGAGAAGTCTGgCGTTAgTGCCCqaattccqAtcG	400
<u>TcAACcac</u>	

(SEQ ID NO: **)

FIGURE 19

5-8
CACGTTTCGTGTTTCGGAGCCGCTTTAACCCACTCTGTGGAAG
GTGCAAAGCACAAGCCTCGGCGAAATTGGGTGAGACACCTTC
5-10

(SEQ ID NO:)

FIGURE 20

1	<u>MAPKKRKRVG</u>	<u>IHGVPAAAE</u>	RPFQCRICMR	<u>NFSRSDNLSE</u>	HIRTHTGEKP	FACDICGRKF
61	<u>ARNAHRINHT</u>	<u>KIHTGSQKPF</u>	QCRICMRNFS	<u>RSDTLSEHIR</u>	THTGEKPFAC	DICGRKFAAR
121	<u>STRTHHTKI</u>	<u>LRQKDAARG</u>	QLVKSELEEK	KSELRHKLKY	VPHEYIELIE	IARNSTQDRI
181	LEMKVMEFFM	KVYGYRGKHL	GGSRKPDGAI	YTVGSPIDYG	VIVDTKAYSG	GYNLPIGQAD
241	EMQRYVEENQ	TRNKHINPNE	WWKVYPSSVT	EFKFLFVSGH	FKGNYKAQLT	RLNHITNCNG
301	AVLSVEELLI	GGEMIKAGTL	TLEEVRRKFN	NGEINF		

(SEQ ID NO: **)

FIGURE 21

1	MAPKKKRRKVG	IHGVPAAAE	RPFQCRICMR	NFSRSDSLSR	HIRTHTGEKP	FACDICGRKF
61	ADSSNRKTHT	KIHTGGGGSQ	KPFQCRICMR	NFSRSDSLSV	HIRTHTGEKP	FACDICGRKF
121	ADRSNRITHT	KIHLRQKDAE	RGSQLVKSEL	EEKKSELRHK	LKYVPHEYIE	LIEIARNSTQ
181	DRILEMKVME	FFMKVYGYRG	KHLGGSRKPD	GAITYVGSP	DYGVIVDTKA	YSGGYNLPIG
241	QADEMQRYVE	ENQTRNKHIN	PNEWWKVYPS	SVTEFKFLFV	SGHFKGNYKA	QLTRLNHITN
301	CNGAVLSVEE	LLIGGEMIKA	GTLTLEEVR	KFNNGEINF		

(SEQ ID NO: **)

FIGURE 22

CGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCATGGTGAG
CAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACG
TAAACGGCCACAAGTTCAGCGTGTCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAG
CTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGGCCACCCTCGT
GACCACCCTGACCTACGGCGTGCACTGCTTCAGCCGCTACCCCGACCATGAAGCAGC
ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTC
AAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGT
GAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACA
AGCTGGAGTACAACATAACAGCCACAACGTCTATATCATGCCCCGACAAGCAGAAGAAC
GGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCACTCGC
CGACCACTACCAGCAGAACACCCCATCGGGCAGCGCCCGTGCTGCTGCCCCACAACC
ACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATG
GTCTGTGAGTTCGTGACCGCCCGGGATCACTCTCGGCATGGACGAGCTGTACAA
GTAAAGCGGCCGCACTCTAGATCATAATC

FIGURE 23

CGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCATGGTGAG
CAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACG
TAAACGGCCACAAGTTCAGCGTGTCGGGCGAGGGCGAGGGCGATGCCACCTACGGCAAG
CTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGGCCCACCCTCGT
GACCACCCTGACCTACGGCGTGCACTGCTTCAGCCGCTACCCCTAACACGAAGCAGCAC
GACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAA
GGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGA
ACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAG
CTGGAGTACAACACTACAAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGG
CATCAAGGTGAAGTTCAGATCCGCCACAACATCGAGGACGGCAGCGTCGAGCTCGCCG
ACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGACAACCAC
TACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACAATGGT
CCTGCTGGAGTTCGTGACCGCCGCGGGATCACTCTCGGCATGGACGAGCTGTACAAGT
AAAGCGGCCGCGACTCTAGATCATAATC

FIGURE 24

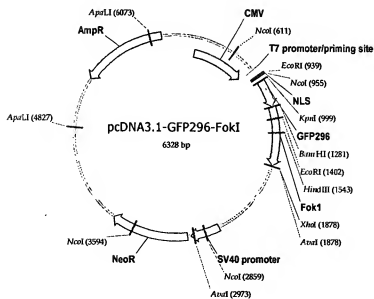
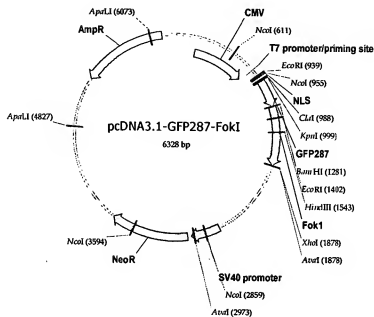


FIGURE 25

287a-F1/296s-F1

Uncut control
Marker



FIGURE 26

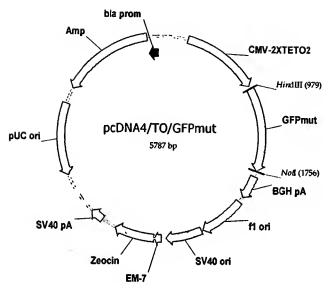


FIGURE 27

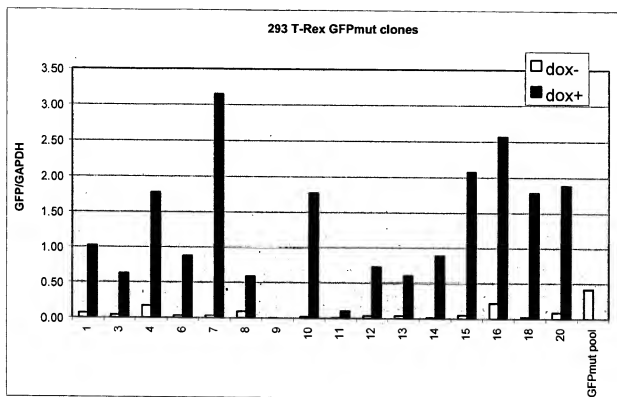


FIGURE 28

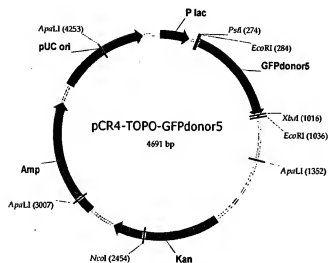


FIGURE 29

GGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAA
CGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGA
CCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCGTGCCCTGGCCACCCCTCGTGACC
ACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGA
CTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGG
ACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCCTGGTGAAC
CGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCT
GGAGTACAACACTACAAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCA
TCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGAC
CACTACCAGCAGAACACCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGACAACCACTA
CCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCC
TGCTGGAGTTTCGTGACCGCCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA
AGCGGCCGCGACTCTAGATCATAATC

FIGURE 30

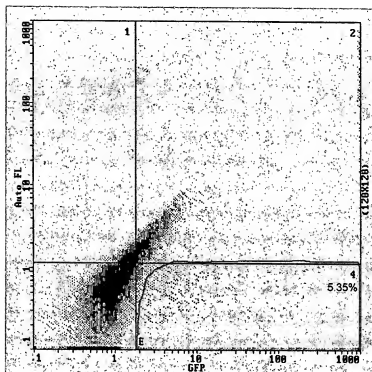


FIGURE 31

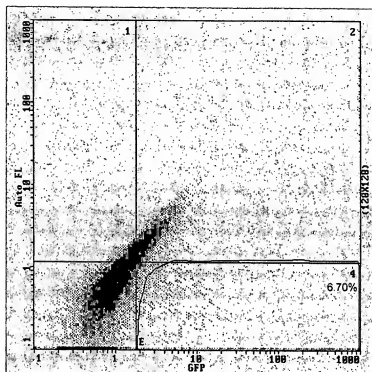


FIGURE 32

GGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAA
CGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGA
CCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCACCCCTCGTGACC
ACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGA
CTTCTTCAAGTCCGCCATGCCCCAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGG
ACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAAC
CGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCT
GGAGTACAACACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCA
TCAAGGTGAAGTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGAC
CACTACCAGCAGAACACCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGACACCCTA
CCTGAGCACCCAGTCCGCCCTGAGCAAGACCCCAACGAGAAGCGCGATCATATGGTCC
TGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA
AGCGGCCGCTCGAGTCTAGAGGGCCCCGTTAAACCCGCTGATCAGCCTCGACTGTGCCT
TCTAGTTGCCAGCCATCTGTGTTTGCCCTCCCCCGTGCCCTCCTTGACCTGGAAGG
TGCCACTCCCACTGTCTTTTCTAATAAAATGAGGAAATTGCATCGCATTTGTCTGAGTA
GGTGTCAATCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA
GACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGCGGGAAGAAG
CAGCTGGGGCTCTAGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGG
GTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCT
TTCGCTTTTCTCCCTTCTTCTCGCCAGTTGCGCGGCTTTCCCGTCAAGCTCTAAA
TCGGGGGCTCCCTTTAGGGTTCCGATTAGTGCTTTACGGCACCTCGACCCCAAAAAA
TTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCT
TTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCACAACTGGAACAACACT
CAACCCATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTTCGGCTATT
GGTTAAAAAATGAGCTGATTAAACAAAAATTAACGCGAATTAATCTGTGGAATGTG
GTCAGTTAGGGTGTGGAAGTCCCAGGCTCCCAGCAGGCAGAAGTATGCA

FIGURE 33

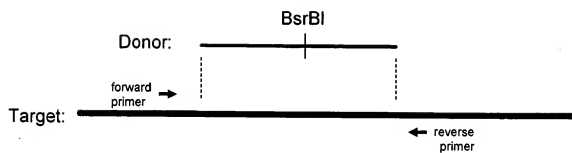


FIGURE 34

